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Project title : Investigations on the development of mutant strains of molds with increased ability to synthesize vitamin B for use in improving the quality of mold-ripened cheese

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INVESTIGATIONS ON THE DEVELOPMENT OF MUTANT STRAINS OF MOLDS WITH INCREASED ABILITY TO SYNTHESIZE VITAMIN B FOR USE IN IMPROVING THE QUALITY OF MOLD-RIPENED CHEESE

Summary

It is well-known a fact that more and more complicated procedures applied in production of foods in many cases are resulting in decrease of their vitamin content. This, particularly at a widespread use of preserves, may cause the deficiency of these important components of human diet which, in some cases, can by no means be equalized by synthetic vitamins. In this connection therefore it seems to be highly purposeful to conduct investigations aimed at increasing of vitamin content in particular foods.

Efforts have been undertaken in the present work to improve the nutritional value of mold-ripened cheese (Camembert and Roquefort-type varieties) through an application to their manufacture of mold strains with possibly high abilities to biosynthesize some B-group vitamins (thiamine, riboflavin, niacin, pantothenic acid, biotin and cobalamin).

On the basis of a comparative appraisal of ten strains of *Penicillium candidum* carried out in cultures grown in milk and Camembert cheese two strains, Nos 2 and 10, have been chosen as the most suitable. These strains secured the best flavor of cheeses and a considerable content of the B-group vitamins in them. In the course of investigations the changes in vitamin content in media, occuring as effect of *Penicillium candidum* mold development, have been characterized with their simultaneous observation in a special self-made device. This device enabling to grow the mold at a steady flow of medium also allowed to conduct investigations in more rigorously controlled conditions, at the same time reducing influence of post-fermentation products on the growth and activities of the mold itself.

These investigations have made possible to carry out a number of observations with regard to effects of some agents on the production of vitamins by *Penicillium candidum*. In media rich in proteins and containing already some quantities of vitamins an intensive increase in niacin content could be observed, whereas other vitamins were utilized during the first stage of mycelium growth and only later excreted into medium. In media with poor composition these phenomena were occuring in quite another way. Besides, a marked effect of medium's pH on the observed changes has been shown.

From the above described investigations conclusions may be drawn with regard to important influence of conditions in which the mold developes on its abilities to produce vitamins. No doubt then that it would be highly desirable to undertake more detailed studies on the most favorable conditions for manufacture of mold-ripened cheese which at the same time could secure their best qualities and nutritional values. This problem, however, by far exceeds the limits of the present work.

In the further stages of investigations 45 mutants of *Penicillium candidum* have been isolated in result of irradiations of the *Penicillium candidum 2* and *10* mycelium suspensions with the U.V., X-, and gamma-rays. As the first criterion for selection has been adopted their technological evaluation. Best qualities of cheese were secured by mutants labelled as Nos 6, 10, 17, 45 and 47. The chosen mutants were then subjected to biochemical appraisal with the simultaneous testing of their abilities for hydrolisis of protein and fat as well as those to biosynthesize the B-group vitamins. On the basis of results gained the *Penicillium candidum* mutant No 10 has been distinguished producing by about 20 per cent more niacin and about 45 per cent more pantothenic acid than its parent strain. Besides, the mutant No 45 was distinguished for its abilities for biosynthesis of thiamine and riboflavin and also the *Penicillium candidum* mutant No 47 producing about 25 per cent

more niacin and about 20 per cent more pantothenic acid than the parent strain. After about one-year storage the *Penicillium candidum* mutants Nos 45 and 47 were subjected to another evaluation in manufacture of Camembert cheese. Experiments have shown that the mutant No 45 has preserved its abilities to produce vitamin B_2 and also intensively synthesized niacin. The mutant No 47, however, after the same storage period has shown decreased abilities to produce vitamins.

As a practical result of investigations on selection of *Penicillium candidum* strains may be regarded the possibility of supplying for practical use of a set of mold strains (three parent strains and one mutant) which, when applied for manufacture of Camembert-type cheeses, will cause the increase of the B-group vitamins in them at a simultaneous improving of their flavor.

The comparative technological and biochemical evaluations of selected seven strains of *Penicillium roqueforti* supplied a basis for choice of the strains Nos 2 and 7 securing good organoleptic qualities of cheese. Besides, the *Penicillium roqueforti* strain No 2 was able to produce more niacin and cobalamin than the other strains and also produced considerable quantities of riboflavin.

From among other strains the *Penicillium roqueforti* strain No 7 has distinguished itself by its abilities to synthesize thiamine and biotin.

The application of mutagenic agents which have been applied in the course of investigations on *Penicillium candidum* has led to selection of mutants Nos "2/2" and "2/5". From among these mutants the *Penicillium roqueforti* mutant "2/5" is able to synthesize over 30 per cent more niacin than its parent strain and about 20 per cent more pantothenic acid, whereas the mutant "2/2" is able to produce more riboflavin. In result of experiments for manufacture of Roquefort cheese can be recommended the mixture of the *Penicillium roqueforti* No 7 and the mutants labelled "2/2" and "2/5".

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I. INTRODUCTION

The transformation of the plant and animal raw materials into food in consequence of complicated technological processes frequently results in decreasing the content of their components having basic importance in human diet. Vitamins are to be listed here on the first place. In most cases these componnds may be considered as constituents of enzymes which are responsible for metabolism in a live organism. Deficiency of vitamins leads to limitation in growth, the decrease of the organism's freedom from inducing of diseases, and to diseases themselves. Cases of vitamin deficiency are reported not only in underdeveloped countries, but also in those with a high standard of living. In the southern states of the U.S.A., for example, 170,000 cases of pellagra were reported in 1917 at the accompanying high death rate of those affected (19).

Thus, all efforts aimed at increasing the nutritive value of various foods should be considered as highly reasonable. It was the purpose of the present work, representing a contribution to these efforts, to increase the vitamin content in Camembert and Roquefort-type cheeses by an application of biochemical agents.

As the molds are considered to be the main agents of vitamin biosynthesis in these cheeses, the author's efforts have been concentrated on selecting of natural and induced mutants of *Penicillium candidum*, being the main component of superficial flora in Camembert cheese, and of *Penicillium roqueforti* developing in Roquefort cheese. To obtain mutagenic effects the irradiations with U.V.-, X-, and gamma-rays have been applied. From a review of subject bibliography a conclusion may be drawn that a considerable increase of the B-vitamins content occurs during the ripening of mold-ripened cheese. As a clear indication of this fact may be taken differences in amounts of vitamins found in a fresh cottage cheese and in ripened cheese, as well as the differences between their amounts in the outer and inner parts of Camembert cheese.

Thiamine. In estimation of the thiamine content in cottage cheese considerable differences in values are reported by various authors. So, for example, Monzini (22) estimates its content for 17 mcg per 100 g, Sobry (30) for 30 to 40 mcg per 100 g, Costavassilis (8) for 90 to 105 mcg per 100 g, and Dluzewski et al. (11) for 37 mcg per 100 g of cheese.

Riboflavin. According to Randoin in the French cottage cheese the content of riboflavin fluctuates from 200 to 400 mcg per 100 g. Damil, Norris et al. (9) and Irvine (4) estimate the content of this vitamin in cottage cheese for 190 to 300 mcg per 100 g, Monzini (22) for 135 mcg per 100 g, whereas according to Antoniani (2) its content amounts to 92 mcg per 100 g only.

Investigations carried out in Poland by Dluzewski, Pijanowski and Zmarlicki (11) have shown that the content of this vitamin in cottage cheese amounts to 410 mcg per 100 g. The average content of vitamin B in Camembert cheese was estimated for 0.83 mg per 100 g by Sullivan (33) and for 0.28 to 0.52 mg per 100 g by Burkholder et al. (4).

Works of Davidov (10), Monzini (22), Burkholder et al. (4) and Cailleau et al. (5) have shown that in the outer parts of Camembert and Brie varieties there is considerably more vitamin B_2 than in the inner ones. According to Burkholder in a sample from the inner part the B_2 vitamin content amounts to 0.28 – 0.52 mg per 100 g, while in portions nearer to the rind – 0.66 to 1.22 mg per 100 g.

Niacin. It is reported by Davidov (10) that only 10 per cent of the milk niacin remains in cheese while the remainder flows off with the whey. However, Reif et al. estimate the percentage of niacin remaining in the cottage cheese mass as high as for about 23 per cent. According to the above-named authors the remaining niacin content in cottage cheese amounts in average to 71 mcg vitamin PP per 100 g. Nevertheless, in effect of biosynthesis of this vitamin by microorganisms a considerable increase of its content during the ripening process may be observed.

The content of vitamin PP in cheese varies widely and is much depending on the manner in which the cheese is handled and also on the kind of microorganisms active in fermentative processes. Differences are recorded between the separate samples taken from the same cheese and between the mean values determined for their different types as well. Opinions as to the content of this vitamin in the hard and soft cheese are also widely differing. Some authors (25, 10, 4, 5, 12) report a low level of this vitamin, whereas according to others (7, 12) the levels are considerably higher. However, there is a complete agreement between different authors as to the fact that the content of vitamin PP in soft cheese is higher.

Sullivan (33) estimates the content of this vitamin for 1.24 to 1.60 mg per 100 g, Cailleau (5) and Claveau (7) for 0.70 to 0.95 mg per 100 g, while Burkholder (4) for 0.005 to 0.27 mg per 100 g only. In one of his recent works Shahani et al. (31) after testing 10 samples of Camembert cheese have stated that the content of vitamin PP was ranging as widely as from 0.046 to 1.24 mg per 100 g (0.59 mg per 100 g being the mean value) with a standard deviation amounting to 414.1. The above-named authors are of opinion that the soft cheeses, characteristic of their greater proteolytic changes during the ripening, are showing a higher level of this vitamin in comparison with the hard, semi-hard and cottage cheese. Many authors (4, 33, 5, 7, 29) report that in the outer parts of Camembert cheese the niacin content is considerably greater than in the inner ones. According to Burkholder (4) in the inner parts of cheese the niacin content amounts to 0.35 mg per 100 g, while in outer parts as high as to 2.3 mg

per 100 g. Ritter (28) and Roehrlich (29) expressed an opinion that both yeasts and *Oidia* developing on the surface of cheese are synthesizing vitamin PP, while the lactic acid bacteria are utilizing considerable amounts of this vitamin, especially in a logarythimc phase of their growth. According to Roehrlich (29) after inhibiting the growth of lactic acid bacteria in Camembert cheese a considerable increase of this vitamin content can be observed in effect of the yeast and *Oidia* action, but in the final stage the content of vitamins decreases slightly as result of their utilization by *Micrococci* and *Corynebacteria*.

Pantothenic acid is present in milk in considerable but varying quantities. Its average content amounts to about 200 mcg per 100 ml of milk, but differences between the milks from various cows may range as widely as from 100 to 500 mcg per 100 ml. It has been found that a greater content of pantothenic acid is usually accompanied by a higher level of biotin.

Pasteurization does not necessarily result in essential changes in the content of pantothenic acid. The same is true with regard to hydrogen peroxide addition. After separation of whey the remainder of this vitamin in a fresh cheese amounts to about 40 per cent (34).

The scarce reports relating to the content of this vitamin in cheese have been supplemented by a comprehensive work by Shahani et al. (31). The authors report that in cheese with proteolytic changes more developed in the course of ripening this content may amount as high as to 1.52 mg per 100 g, in a hard cheese to 0.39, in semi-hard to 0.28, and in the fresh cottage cheese to 0.24 mg per 100 g.

According to Burkholder (4) the pantothenic acid content varies from 0.04 to 1.4 mg per 100 g, while according to Cailleau (5) from 0.09 to 1.00 mg per 100 g, to Sullivan et al. (33) from 0.59 to 0.96 mg per 100 g, to Haudiniere (13) to 0.85, and according to Shahani et al. (31) even from 0.46 to 6.58 mg per 100 g (1.4 mg per 100 g in average) with the standard deviation in 15 Camembert samples ranging approximately to 2,000.

The increase in content of this vitamin in the course of ripening is emphasized by Burkholder et al. (4) and Cailleau et al. (5) and is to be observed mainly on the surface of Camembert. According to Burkholder et al. (4) in the inner parts of Brie cheese the content of pantothenic acid in average amounts to 1.4 mg per 100 g, while in its outer parts to 6.5 mg per 100 g. The magnitude of differentiation in the content of pantothenic acid in these parts according to Cailleau et al. (5) is about threefold. Brochu et al. (3) have found that the lactic acid bacteria in the initial stage of cheese ripening are utilizing some quantities of this compound for their own growth. It has been shown by Ritter that *Oidium lactis* when grown in a pantotheneless medium is able to synthesize 170 to 190 mcg of pantothenic acid per 100 ml.

Biotin. According to Shahani et al. (31) the content of biotin in strongly proteolytic cheeses amounts in average to 2.51, in the hard cheese to 1.50, in semi-hard cheese to 1.48, and in the raw cottage cheese to 1.85 mcg per 100 g. In 20 samples of Camembert this content varied from 1.92 to 17.87 mcg per 100 g. The biotin content in the fresh cheese is estimated as high as for 1.2 mcg per 100 g, while in the ripened Camembert for 5.6 mcg per 100 g. Differences in biotin content observed in various parts of cheese are considerable. In the inner parts they amount from 0,002 to 7.6 mcg per 100 g, while in those nearer to the rind from 2.4 to 63.3 mcg per 100 g. According to Brochu et al. (3) the biotin content in the inner parts of mold-ripened cheese amounts to 2.52 and in outer parts to 6.92 mcg per 100 g. Burkholder (4) observes a considerable rise of biotin level in Camembert cheese during its ripening. Within 44 days this level has risen from 4.0 to 34.5 mcg per 100 g. Ritter (26) has characterized the abilities of *Penicillium candidum, Penicillium glaucum, Oidium lactis* and the yeast strains for biosynthesis of biotin as high as for 0.035 to 0.95 mcg per 100 ml of the biotinless medium.

Vitamin B_{12}. As it was early stated pasteurization does not result in apparent decrease of cobalamin content in milk. In the course of acid clotting of milk 44 per cent of vitamin B_{12} is usually passing into whey, while in effect of rennet clotting this percentage amounts to 60% (2). Since this vitamin is bound with protein in milk a greater passing of vitamin B_{12} into the whey in effect of rennet clotting is explained by Collins et al., among others, by the fact that in the course of this process the enzymatic separation of cabalamin from its protein carrier occurs. Data reported by various authors and relating to the content of vitamin B_{12} in cheese are different what, according to Karlin (16), may chiefly be explained by the application of different analytical methods.

Using microbiological method with *Lactobacillus leichmanii*, Collieri (6) estimates its content in Camembert cheese for 1.15 mcg per 100 g, Lichtenstein et al. (with *Ochromonas malhamensis*) as high as for 0.59 to 0.66 mcg per 100 g, while Karlin (15) for 1.45 (with *Propionibacterium leichmanii*), and Ritter (27) (with the strain *Bacterium coli* 113–3) for 1.15 mcg per 100 g. The B_{12} vitamin content in non-ripened cheese manufactured in France (15) is estimated for 0.65 to 2.0 mcg per 100 g and it can be generally stated that only the slight deviations from the above value have been found. The increase in content of vitamin B_{12} in Swiss cheese during the ripening may be considered as result of its biosynthesis by propionic acid bacteria.

The content of many vitamins belonging to the B-group in the Roquefort-type cheese is greater than that in cottage cheese what may be regarded as an evidence of their biosynthesis by microorganisms developing in this cheese. According to Lie and Lunde (20) the thiamine content in Roquefort cheese is estimated as high as for 200 to 375 mcg per 100 g of cheese. Adrian and Levy

(5) estimate the riboflavin content for 700 to 900 mcg per 100 g.

The average niacin content is estimated (31) for 629 mcg per 100 g at a considerable differentiation of results obtained from analyses carried out on different samples and their extreme values varying as widely as from 84 to 1.044 mcg per 100 g. The biotin content is estimated by Shahani et al. (31) for 1.49 mcg per 100 g (1,02 to 2.81), while according to Ritter (27) it amounts in average to 3.6 mcg per 100 g. The folic acid content is estimated (31) for 49.0 mcg per 100 g (20 to 82), but according to Karlin (17) for 37 mcg per 100 g only, whereas cobalamin content for 0.65 mcg per 100 g of cheese.

From the above data may be seen that the content of the B-group vitamins is increasing in the mold-ripened cheese, what may be additionally testified by information from several publications stating, that the vitamin content is much higher in the cheese mass parts nearer to the mold. When comparing values given for the fresh cottage cheese with those for mold-ripened cheese the following approximate increase in vitamin content in Camembert cheese may be stated: thiamine – sevenfold, riboflavin – twofold, niacin – sixfold, pantothenic acid – sixfold, biotin – 1.5 times, with no increase in the cobalamin content at all.

The values for Roquefort cheese are as follows: vitamin B_1 – sixfold, vitamin B_2 – twofold, vitamin PP – twofold, pantothenic acid – twofold, biotin – threefold.

It should be emphasized, however, that the works carried out up to now are mainly related to the static evaluation of vitamin content in different types of cheese and in some cases to differences occuring within the various stages of manufacture. No works with the view to increasing the vitamin content in cheese by means of modification in technology or selecting the strains with higher abilities for biosynthesis of vitamins have been recorded as yet.

This latter problem constitutes the objective of the present work. However, the success of investigations conducted was much depending on the correctness of the following hypotheses:

1) That the mold constitutes the main agent influencing the increase of the B-group vitamin content in the Camembert and Roquefort-type cheeses.

2) That there exist considerable differences between the strains of the same species with regard to their abilities for biosynthesis of vitamins.

3) That in effect of action of mutagenic agents it is possible to obtain mold mutants with the increased vitamin production abilities if compared with those belonging to the parent strains.

Thus, before the passing to technological evaluation, an empiric justification of correctness of the above hypotheses should be presented.

II. METHODS OF INVESTIGATION

1. SCHEME OF EXPERIMENTS

Vitamin Determinations

The first stage of experimental work covered the tests carried out with the purpose to show the ability of *Penicillium candidum* and *Penicillium roqueforti* strains for biosynthesis of the B-group vitamins. These tests were carried out in the Capek-Dox synthetic medium, in milk and in the cheese mass. In effect of a comparative examination of different strains of the same species the populations with the most appropriate technological and biochemical properties were selected. Sensory testing of cheese manufactured with the use of a given strain was applied as a basis for technological evaluation.

The range of biochemical investigations covered the determination of vitamin content in a medium and also determination of their proteolytic and lipolytic properties.

In the second stage the tests were conducted with the aim to obtain mutants in effect of such mutagenic treatment as irradiation with U.V.-, X-, and gamma-rays and also the treatment with some chemical agents. Independently from the above the preliminary tests were carried out to evaluate the influence of some physico-chemical factors as well as the medium's composition on the abilities of *Penicillium candidum* for synthesis of vitamins.

2. ANALYTICAL METHODS

Vitamins were determined with the use of microbiological methods in accordance with recommendations given in special technical booklets issued by the French National Research Center (1).

/A/ The thiamine content was determined with the use of the strain *Lactobacillus fermenti 36 (ATTC 9338)* in the Sorret and Cheldelin medium (35). Growth of the strain was observed nephelometrically after the 18-hour incubation at 37°C. The cheese samples were at first ground in mortar with a water addition to obtain 10% suspension. Both the suspension and the milk were hydrolized by physical and enzymatic agents to liberate the bound thiamine. The sample was dissolved by addition of 15 times as great volume of 0.1 N H_2SO_4 then heated with the flowing steam for 30 min., and after cooling its pH brought to 4.5 with 2 M natrium acetate. Further on, the sample was hydrolized overnight with papain and takadiastase at 37°C. The protein precipitated at pH 4.5 was separated by filtration. After filling to a given volume the sample was twice shaken with an addition of 1:3 petrol ether and ethylic ether mixture.

/B/ For determining of riboflavin *Lactobacillus casei (ATTC 7469)* and the test medium proposed by Snell and Strong (32) were used. The growth rate of the strain was determined by measuring the acidity of medium after 72-hour incubation at 30°C. After extraction of fat with 3:1 mixture of ethylic ether and petrol ether the sample was dissolved in a 10 times as great volume of 0.1 N NaCl, heated for 30 min. at 120°C and its pH brought to 4.5 with natrium acetate. It was hydrolized with the mixture of papain and takadiastase for 15 hours at 45°C, filtered and then filled to a given volume and its pH brought to 6.8 against bromethymol blue.

/C/ Niacin was determined with the use of the strain *Lactobacillus arabinosus 17/5(ATTC 8014)* and the medium according to Snell and Wright (1). The strain applied allowed for determination of 0.05 to 0.5 mcg of this vitamin. Growth of the strain in medium was determined by titration of fermenting solution with 0.1 N NaOH after the 72-hour incubation at 37°C. Vitamin was extracted from the sample by acid hydrolisis. To the cheese or milk suspension 5 times as great volume of 0.2 N H_2SO_4 was added and the mixture heated for 30 min. at 120°C. Proteins precipitated at pH 4.6 were separated by filtration and the filtrate brought to pH 6.8.

/D/ Pantothenic acid. For this test as the test strain *Lactobacillus arabinosus 17/5(ATTC 8014)* and as the medium a set proposed by Skeggs and Wright (1) have been applied. The medium was titrated after a 72-hour incubation at 37°C.
Vitamin extraction: water emulsion was brought to pH about 6.0 and heated for 15 min. at 120°C, then pH was brought to 4.5 and the enzymatic hydrolisis carried out by means of mylase-P at 30°C for about 16 hours. According to Unna and Mushett the efficiencies of both entylpantothenate and enthylacethylpantothenate proved to be equal to that of pantothenic acid in biological investigations carried out in rats and chickens, while in tests on *Lactobacillius casei* they have shown only 4 to 20 per cent of their previous efficiencies. The pantothenic alcohol, which was equally active in tests carried out in rats (1) as the vitamin itself, has shown its inhibitive activity when used on *Lactobacillus casei* (1).

/E/ The biotin was determined with the use of strain *Lactobacillus arabinosus 17/5(ATTC 8014)* grown in the Wright and Skeggs medium (1). Vitamin was extracted from the sample by acid hydrolisis. To 1 ml sample or water suspension of cheese 5 ml 3 N H_2SO_4 were added and the sample heated for an hour with the flowing steam. The incubation of several inoculated solutions was carried out at 30°C during 72 hours with a simultaneous determination of sugar fermentation efficiency by titration with 0.1 N NaOH.
/F/ For determining of cobalamin the microbiological method was applied using the *Coli aerogenes 113–3* mutant as the test strain.

CHEMICAL ANALYSES

/A/ The determination of the total solids of mycelium was carried out by drying the sample ground with sand to constant weight at 105°C.

/B/ The determination of proteolytic properties of mold in a series of preliminary comparative tests and during the growing of mold in a liquid medium was based on alcoholic titration of fermented medium. For this purpose a method proposed by Masek et al. (21) was used. The sample was titrated with 0.1 N NaOH in 50% and 90% solutions of ethylic alcohol.
/C/ Proteolytic changes in cheese. The examination of proteolytic changes in cheese was based on determination of amino acids in a filtrate obtained after the precipitation of protein from an aqueous extract with sodium phosphotungstate (35). Results were expressed in terms relating to the total nitrogen content in cheese. Nitrogen was determined by the Kjeldahl method.
/D/ Lipolytic activity. The fat for analysis was obtained by melting suitably prepared cheese samples.
No solvents were used. About 100 g cheese were ground in a porcelain mortar with about 100 ml of water at 30°C to 35°C. The solution obtained was transferred to Erlenmeyer flask of 300 to 500 ml volume. The shaking for 5 minutes caused the separation of lipid fraction from the remaining constituents. The upper layer was then transferred to test tube and after heating to 40 – 50°C centrifuged at 3,000 rpm for 10 min. The separated lipids were filtered through a filter paper into bottles of dark-colored glass. The lipid samples were analysed or stored under refrigeration up to 2 days. Lipid acidity was determined by titration with 0.1 N NaOH of a lipid sample weighing 2.00 to 2.50 g diluted in 25 ml of a 1:1 neutral ether-alcohol mixture against phenolphtalein. The result was expressed in ml 0.1 N NaOH used for neutralizing 100 g of lipid.
/E/ The peroxide number was determined by the Lea method and result expressed in milliequivalents of oxygen per 1 kg of lipid (Lea number x 2) (18).
/F/ 2-thiobarbituric acid test /TBA/. This test served for determining the quantity of products of further oxydative rancidity of lipids, principally of betta-keto-aldehydes (23).
/G/ The examination of lipolytic activity of molds grown in a liquid medium was carried out in the following manner: the stationary cultivation of molds was conducted on hydrolized milk prepared according to recommendations of Bogdanov (21) with 5% addition of butterfat. Incubation period – 21 days. Samples were determined by the Kettstorfer method.
/H/ The pH of fermentative liquids was determined by the use of glass and calomel electrodes in pH-meter "Radiometer 22 – Copenhagen" of Danish manufacture.
/I/ The potentiometric titration was carried out by the use of the above-mentioned pH-meter.

3. THE MANNER OF SPORE SUSPENSION PREPARATION AND THE METHOD OF INOCULUM PREPARATION AND CULTIVATION

The molds were grown on slants of the wort agar at 20°C for three weeks. After that period spores were rinsed with the physiological solution of salt. The mycelium was separated by filtration through the sterile cotton wool. Concentration of spores was determined by a direct microscopical counting in the Thom chamber. The solution was then standardized to the content of 1:2,000,000 spores per 1 ml. Inoculum constituted 1 ml of the above-mentioned suspension in the medium under examination.

III. RESULTS

1. CHARACTERISTICS OF SELECTED STRAINS OF PENICILLIUM CANDIDUM

Industrial strains of *Penicillium candidum* applied in various countries for manufacture of the Camembert-type cheese constituted the object of studies and were numbered according to their origin as follows: Nos 1 and 2 – strains from the USSR, No 3 – from Switzerland, No 4 – from Netherlands, No 5 – from Hungary, No 6 – from New Zealand, No 7 – from the GDR, No 8 – from France, No 9 – from Czechoslovakia, and No 10 – from Denmark. Pure cultures were grown in the wort-agar medium and inoculated once a month.

/A/ **Proteolytic and lipolytic properties.** Four series of tests in mold cultures were carried out on the reconstituted milk. Already preliminary observations have shown that the growth of mycelium in a liquid medium was not uniform. A modification has been introduced by means of clotting the milk with 1:20,000 rennet solution previously sterilized by filtration through the bacteriological filters. The superficial layers of coagulated milk were inoculated with a suspension of *Penicillium candidum* spores with a density of about 12,000,000 per 1 ml and then incubated for 21 days. After the 10-day incubation period the mold mycelium has grown on the surface of medium and the process of peptonization of milk protein could be observed. After the 21-day incubation this process usually led to clarification of the medium. Mycelium formed a dense, folded "skin" on the surface. The growth rate of mycelium was different in particular strains. After the 3-week incubation period, however, differences in a dry solid of mycelium were so insignificant (see Table 1) that in view of comparatively large variations between results obtained from the successive series of experiments they could be regarded as negligible. Only the strain No 6 has distinguished itself by a greater increase in the mass of mycelium.

The proteolytic changes in milk were accompanied by the increase of pH, amounting to about 8.3. *Penicillium candidum* alkalises the medium rather to a lower extent, namely up to pH 8.0. The growth of molds in milk with a higher initial pH (6.8) has only a very slight effect on the limits of medium alkalization (up to 8.5).

Potentiometric titrations of medium after the 5, 10, 15 and 21-day periods of mold growth have shown that the buffer capacity of hydrolized milk increases in time. It can be assumed therefore that the increase of pH in cheese is hampered by the buffer capacity of medium and also by utilization of lactic acid by the molds and yeasts. Significant differences were found in proteolytic properties of strains under investigation. First of all they are indicated by the content of free amino acids in the postfermentation liquid determined with the use of technical method (Masek et al.) as well as the Kjeldahl micromethod. Figures given in Table 2 (row 3) represent the volume of 0.1 N NaOH in ml used for neutralization of polypeptides shown in its row 6. The proportion (in %%) of amino acids and polypeptides is presented to show the range of proteolytic changes.

TABLE 1

CHANGES IN MILK CAUSED BY STRAINS UNDER INVESTIGATION

Strain	Number of successive trials	Average mass of mycelium	Standard deviation of particular results
Penicillium candidum No 1	4	5.55	0.79
Penicillium candidum No 2	4	5.58	0.93
Penicillium candidum No 3	4	5.55	0.48
Penicillium candidum No 4	3	5.24	0.46
Penicillium candidum No 5	4	5.12	0.357
Penicillium candidum No 6	4	6.16	0.05
Penicillium candidum No 7	4	5.08	0.42
Penicillium candidum No 8	4	5.54	0.49
Penicillium candidum No 9	4	5.35	0.42
Penicillium candidum No 10	3	5.10	0.07

TABLE 2

THE LIPOLYTIC AND PROTEOLYTIC PROPERTIES OF PENICILLIUM CANDIDUM STRAINS

Strain	Kettstofer's number	Amino acids (A) ml 0.1 N NaOH per 100 ml	Amino acid N mg/%	Poly-peptides (P) ml $\frac{N}{10}$ NaOH per 100 ml	$\frac{A}{P+A} 100$ %
Penicillium candidum No 1	47.2	20.7	60.1	19.6	51.4
Penicillium candidum No 2	47.4	25.5	98.8	15.2	62.7
Penicillium candidum No 3	37.2	36.5	113.0	18.5	66.4
Penicillium candidum No 4	53.6	45.6	108.6	21.9	67.6
Penicillium candidum No 5	37.0	36.6	94.8	24.9	59.5
Penicillium candidum No 6	27.6	14.3	58.8	16.3	46.7
Penicillium candidum No 7	51.6	35.9	86.5	20.2	64.0
Penicillium candidum No 8	27.2	20.5	73.7	17.7	53.7
Penicillium candidum No 9	45.9	42.9	135.5	16.2	72.7
Penicillium candidum No 10	42.3	41.4	109.8	12.1	77.4

The Table 2 shows the mean values of four series of experiments. The strains investigated differ essentially as to their lipolytic properties. The mean values representing volumes of 1 N NaOH used for neutralization of 100 g of butterfat (Kettstorfer's number) are given in row 2.

Even after a superficial review of these results different properties of the *Penicillium candidum* strain No 6 become apparent. This strain is characteristic of its lower ability to hydrolize the fats and proteins. On the other hand Nos 9 and 10 are the most active strains. Differences in proteolytic properties of remaining strains are smaller, however. in view of results obtained from the variation analysis they are essential.

/B/ **Ability for biosynthesis of vitamins.** Results relating to the vitamin content in milk after the 3-week fermentation period are presented in Table 3. Here the mean values obtained from the four series of trials (except for the strains Nos 9 and 10 with two series of trials each) are given. A comparative survey of values obtained from a series of successive determinations carried out on one parent strain *Penicillium candidum No 1* (results are given in Table 4) and of the magnitude of standard deviation in population, i.e. of values characterizing the content of the separate vitamins accumulated in the medium in effect of various strains action, points to the essentiality of differences between the various tested strains with regard to their ability for biosynthesizing of vitamins. A statistical evaluation was undertaken with the purpose to justify a hypothetical problem:

TABLE 3

CONTENT OF VITAMIN B IN FERMENTED MILK

Strain used for production	Vitamin B_1 mcg per 100 ml	Vitamin B_2 mcg per 100 ml	Vitamin PP mcg per 100 ml	Pantothenic acid mcg per 100 ml	Biotin mcg per 100 ml	Vitamin B_{12} mcg per 100 ml
Penicillium candidum No 1	38.0	187	2,130	173	2.5	0.17
Penicillium candidum No 2	40.4	209	3,340	210	3.7	0.16
Penicillium candidum No 3	41.7	242	2,580	160	3.6	0.14
Penicillium candidum No 4	40.0	209	2,810	158	3.5	0.13
Penicillium candidum No 5	42.5	225	4,460	130	1.7	0.13
Penicillium candidum No 6	41.7	87	674	154	3.2	0.13
Penicillium candidum No 7	42.2	125	1,220	190	3.5	0.13
Penicillium candidum No 8	39.2	159	1,320	183	2.9	0.14
Penicillium candidum No 9	36.7	287	3.320	196	4.6	0.20
Penicillium candidum No 10	27.6	154	3,315	177	3.5	0.20

are these variations the same in both populations. This evaluation based on the comparison of values taken from Fischer's distribution tests enables to find out that the differences in biosynthesic abilities of strains under examination were significant. From among the strains investigated *Penicillium candidum No 6* is characteristic of its greatest rate of increase of the mycelium mass, its lowest proteolytic activity and its poorest abilities to produce vitamins. The *Penicillium candidum strains Nos 2* and *9* are securing a highest content of the B-group vitamins in milk.

TABLE 4

UNIFORMITY OF RESULTS FROM A SERIES OF PARALLEL DETERMINATIONS OF VITAMINS SYNTHESIZED IN MILK BY THE PENICILLIUM CANDIDUM STRAIN No 1

Test No	Vitamin B_1 mcg per 100 ml	Vitamin B_2 mcg per 100 ml	Vitamin PP mcg per 100 ml	Pantothenic acid mcg per 100 ml	Biotin mcg per 100 ml	Vitamin B_{12} mcg per 100 ml
1	38.2	193	2,300	184	2.46	0.18
2	35.7	146	1,930	175	3.19	0.13
3	36.1	170	1,550	160	3.06	0.19
4	38.5	200	1,870	143	2.49	0.15
5	39.1	206	2,370	169	2.36	0.14
6	35.4	144	1,210	166	2.07	0.17
7	36.7	149	1,360	183	2.29	0.17
8	39.7	159	1,890	175	3.02	0.14
9	37.9	225	2,820	159	2.72	0.15
10	40.7	198	2,500	179	2.03	0.18

For technological evaluation of strains investigated three series of Camembert cheese manufactures were carried out. After the 3-week ripening period the samples underwent sensory tests and analyses of the B vitamin content. Results of analyses expressed in mean values are presented in Table 5. The analysis of preliminary results from which the mean values given in Tables 3 and 4 were obtained reveals considerable differences in successive determinations of vitamins

The differences in abilities to produce vitamin PP were found significant while comparing the particular strains (up to fivefold). The abilities of particular strains of *Penicillium candidum* to produce vitamin B_{12} and biotin are also considerably differing (up to about threefold). A comparison of the vitamin content in the liquid and solid media to select most active molds has proved to be impossible.

The strains have therefore been listed according

TABLE 5

VITAMIN CONTENT IN CAMEMBERT CHEESE

Strain used for production	Vitamin B_1 mcg per 100 g	Vitamin B_2 mcg per 100 g	Vitamin PP mcg per 100 g	Pantothenic acid mcg per 100 g	Biotin mcg per 100 g	Vitamin B_{12} mcg per 100 g
Penicillium candidum No 1	150	790	1,150	850	7.2	1.1
Penicillium candidum No 2	250	930	850	570	7.5	1.15
Penicillium candidum No 3	100	680	780	980	8.5	1.2
Penicillium candidum No 4	70	910	1,200	990	6.4	1.0
Penicillium candidum No 5	430	840	910	300	5.5	0.75
Penicillium candidum No 6	190	880	760	200	3.7	1.3
Penicillium candidum No 7	260	830	730	300	2.8	1.7
Penicillium candidum No 8	200	950	820	880	8.3	1.75
Penicillium candidum No 9	50	870	870	470	8.0	1.7
Penicillium candidum No 10	120	1,070	770	690	3.1	2.1

B_1 and B_2 and of pantothenic acid as well. This observation may be mainly related to trials carried out on a liquid medium. These differences have probably been caused by destructive effect of pH in fermented milk whose pH, as early mentioned, exceeded 8. However, in cheese showing a much higher buffer capacity the pH after the 3-week ripening period remained in a range from 5.9 to 6.3.

to their abilities for vitamin biosynthesis separately with regard to a liquid medium and to cheese. However, their final position on the list has been decided after finding the mean grading as result of evaluation in both media. This made possible to distinguish the strain No 9 securing the greatest content of vitamin B_2, PP, biotin and B_{12} in the medium. The strains Nos 10 and 2 can also be listed in the category of those active, whereas

Nos 7 and 6 are characteristic of their poorest activity.

A comparison of strains listed on the extreme places allows to state that *Penicillium candidum No 9* producing the largest quantities of vitamin B_2, biotin and B_{12} should be listed last with regard to its ability for producing thiamine. On the contrary, the strain No 7 characteristic of its lowest ability to produce B_2, PP and biotin can be listed as one with the greatest ability for synthesizing of thiamine.

/C/ **Technological evaluation of molds.** For the technological evaluation three series of Camembert cheese manufactures were carried out with the use of pasteurized milk. All manufacturing processes were conducted in accordance with instructions in this respect being in force in Poland. After running the whey cheeses were sprayed with the spore emulsion of mold under investigation. The content of spores in emulsion amounted from 1,000,000 to 2,000,000 per 1 ml. In one non-ripened cheese taken from each series the determination of the vitamin content was carried out immediately after salting. After a 3-week ripening period the cheeses underwent sensory tests by a five-expert panel. These tests resulted in the grading of cheese manufactured with the use of the *Penicillium candidum* strains *Nos 1, 2, 6* and *10* as belonging to the first class. The strain No 9 caused that the cheese became strongly piquant with a slightly bitter taste. The use of strains Nos 2 and 6 caused a mild flavor and the mushroom-like smell of cheese possessing a well-developed mycelium. Cheese produced with the strain No 10 was more piquant and had a slightly bitter taste.

On the account of the above described observations the strains Nos 10 and 2 can be considered as the best ones with regard to their high ability to synthesize vitamins and also to secure a good quality of the cheese.

In view of the fact that the methods used for evaluation of the strain quality based on analyses of milk and cheese are very time-consuming ones, efforts were made to find a more simple method of evaluating the biosynthetic ability of mutants the number of which is greatly exceeding that of the parent molds. To find it out whether the shortening of incubation period will be possible without an influence on the final results, studies have been undertaken with the aim to check whether or not the increase in vitamin content during the first stage of incubation is proportional to their final content after a 3-week period. With this purpose in view the characteristics of the velocity of changes in vitamin content occuring in a medium in the course of fermentation process had to be elaborated. Two series of experiments for determination of amino acids, polypeptides and vitamins in medium have been carried out for this purpose.

2. INVESTIGATION OF EFFECT OF SOME AGENTS ON THE ABILITY OF PENICILLIUM CANDIDUM FOR BIOSYNTHESIZING OF VITAMINS

EVALUATION OF THE PENICILLIUM CANDIDUM STATIONARY CULTURES IN SKIMMILK, RECONSTITUTED MILK AND MILK POWDER – A PRELIMINARY STUDY

The inoculum and medium were prepared in the manner described under the paragraph "Methods of investigation". Three of the ten strains were taken for tests and the analyses were carried out after the 5, 10, 15 and 20-day incubation periods. Results obtained from these tests are depicted in Figure 1. The rate of growth of mycelium, the increase in the amino acids and polypeptides content as well as the changes in content of B_1, B_2 and PP vitamins are presented in Figure 1 (diagrams A, B, C, D, E). An observation of the shapes of separate curves enables to draw several conclusions. It can be seen from the diagram A, for example, that the intensive growth of mycelium ends after about 10 days of development at 18°C. From the results of analysis it can be found that this growth is accompanied by an increase of pH up to almost neutral or even alkaline reaction. In this stage an increase in the amino acid and polypeptide content (diagram B) may be considered as insignificant.

A constant but not regular increase of the B_1 vitamin and PP vitamin content has been observed in medium (diagrams C and E). Unfortunately, however, this smaller vitamin content characteristic for the first stage is not accompanied by their completion of fermentation process. Thus, no reduction in time for the growth of molds while isolating the mutants is possible. However, it should be noted that the curve depicting changes in the B_2 vitamin content in medium has a specific shape (diagram D). This seems to require a detailed explanation.

An intensive growth of mycelium occurs at a simultaneous decrease of the riboflavin content. Further on, between the tenth and the fifteenth day the fermentation causes an increase of the B_2 vitamin content in the medium. This increase, however, breaks down at pH exceeding 7. It seems therefore that the two controversary phenomena occur here: an inactivation of riboflavin in an alkaline medium and a synthesis of this vitamin by *Penicillium candidum*. Results are depending on the velocity of these two reactions. In the course of trials with *Penicillium candidum No 3* a strong decrease in the B_2 vitamin content was observed between the fifteenth and the twentieth day. It is slower with the strain No 5, but with the more active *Penicillium candidum* strain *No 9* even a further increase has been observed.

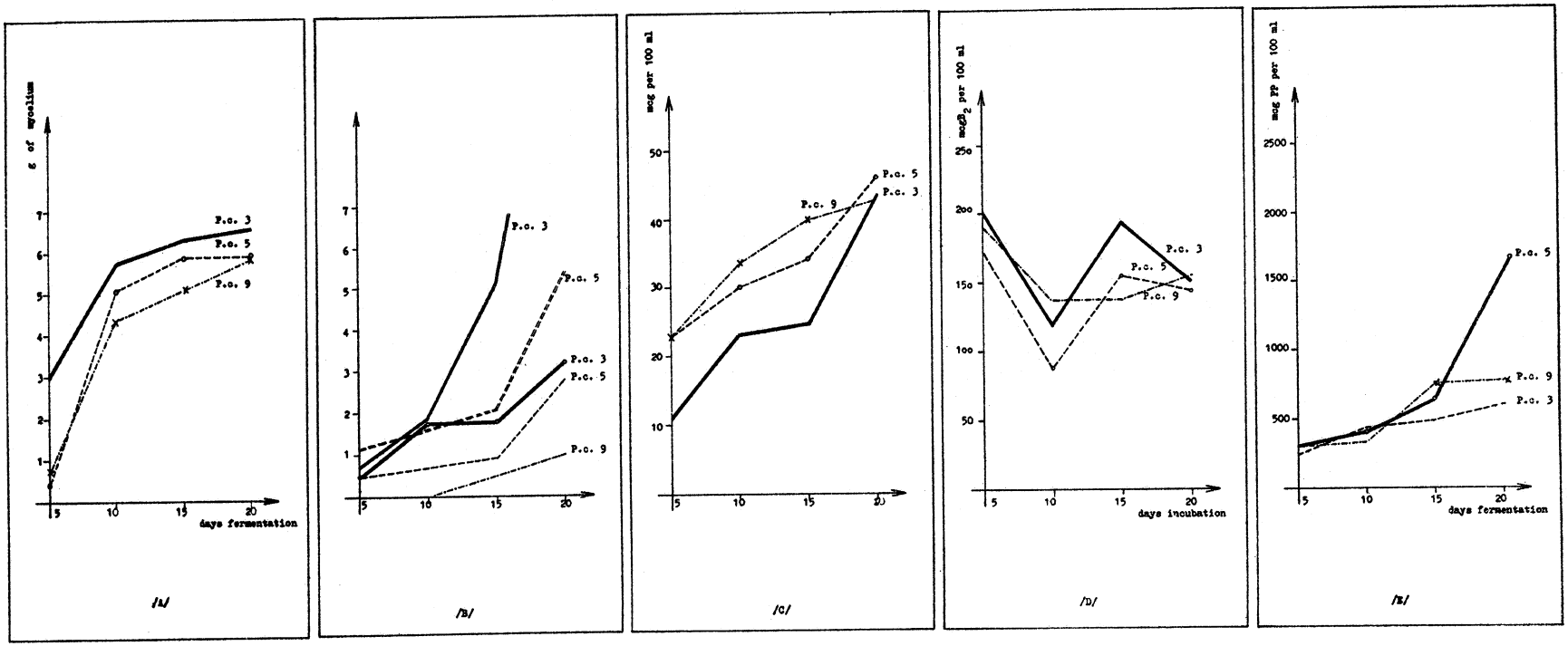


Figure 1.

(A) Growth of mycelium of Penicillium candidum Nos 3, 5 and 9 on skimmilk.

(B) Changes of the amino acids and polypeptides content in milk fermented by the Penicillium candidum strains Nos 3, 5 and 9.

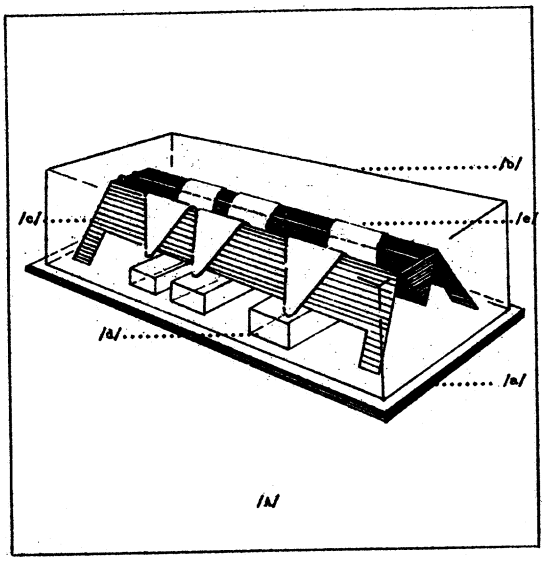
(C) Changes in the thiamine content in skimmilk fermented by various strains of Penicillium candidum.

(D) Changes in the vitamin B_2 content in medium during fermentation.

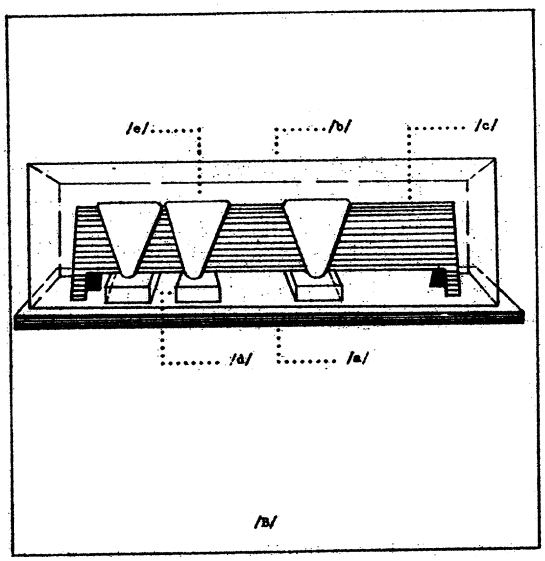
(E) Changes in the vitamin PP content during fermentation of milk under the influence of the Penicillium candidum strains Nos 3, 5 and 9.

Figure 2.

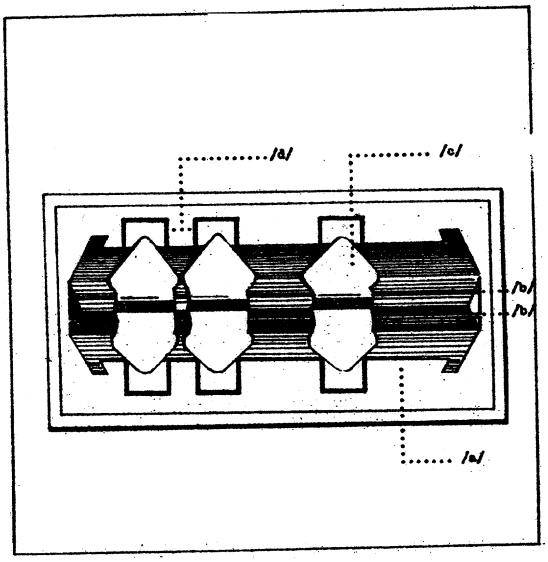
A self-prepared device for culturing the molds.



(A) General view.
(a) Plexiglass base, (b) Plexiglass cover, (c) Stainless steel trough, (d) Plexiglass receptacles, (e) Filter paper strips.



(B) Front view.
(a) Plexiglass base, (b) Plexiglass cover, (c) Stainless steel trough, (d) Plexiglass receptacles, (e) Filter paper strips.



(C) View from above.
(a) Stainless steel trough, (b) Glass rods supporting filter paper strips, (c) Filter paper strips, (d) Plexiglass receptacles.

STUDIES OF EFFECTS OF THE MEDIUM COMPOSITION, pH, AND THE PENICILLIUM CANDIDUM AGE ON BIOSYNTHESIS OF VITAMIN B

(A) Methods of investigation

Culture of molds. Molds were cultured on a sterile filter paper with a flowing medium. Assays were carried out in a self-prepared device which is shown in Figure 2. A small trough made of stainless steel with edges bent outwards so as to bear strips of filter paper is the main part of this device. Strips of filter paper shaped as in Figure 2 (B) and sterilized in an oven are immersed up to the bending point in a water suspension of mold spores and glued to the outer sides of the trough. Strips of the filter paper should be arranged so that a free space is left at the bend where there is no contact with the metal surface.

At the bottom, under the pointed end of the strip a sterilized plexiglass receptacle is put. The entire arrangement is placed under a plexiglass cover, sterilized beforehand with alcohol and U.V.-rays. Medium in the trough is daily supplemented and the post-fermentation liquid drawn for analyses for the pH value and content of vitamins B. Every 2–4 days strips with matured mycelia are collected, rinsed several times in distilled water and dried to a constant weight. The weight of dry paper formerly determined is then subtracted from the weight of dried paper including the mycelium. Incubation period – 12–14 days. Testing was performed on a suspension of spores from a 3-week old culture of *Penicillium candidum* in an agar-broth medium at 25°C.

For analyses were used:

/a/ the Capek-Dox medium with technically pure sucrose (including traces of vitamins),

/b/ 2% solution of peptonized milk (Difco).

Two series of analyses were carried out with the following modifications:

on the Capek-Dox medium at pH 5.0 (4 replications),

on peptonized milk, at pH 4.5,

on peptonized milk, at pH 5.0,

on peptonized milk, at pH 6.0.

Results of analyses were set out in Tables 6, 7, 8 and 9. Under the action of molds the medium whether stationary or flowing turns more alkaline, the pH rising to 8.5. Dynamics of the changes in media are better portrayed by means of diagrams than given as tabulated results.

Diagrams A, B, C and D in Figure 3 represent the relationship between the culture age and the rate of changes of individual vitamin levels and are expressed as percentages of their content in unferemented media.

Levels of vitamins in sterile media are given in the first row of Tables 6, 7, 8 and 9 (time of incubation = 0).

In media deficient in organic components (Capek-Dox) the vitamin content, except for vitamin B_{12}, utilized during the initial phase of development of molds, continuously increases. A most rapid rise occurs in the levels of biotin and pantothenic acid. Concentrations of vitamin B_1 and PP rise throughout the period of examination comparatively slowly to a double amount. The increase in the amount of vitamin B_{12} is negligible and remains within the limits of an experimental error.

Vitamin B_2 synthesizing ability fluctuates in a peculiar manner. During the germination and initial stage of growth of spores riboflavin is utilized, on the fifth day of incubation the rate of biosynthesis rises to drop again and increase markedly towards the end.

Curves in Figure 3, representing the rate of vitamin biosynthesis do not follow a linear pattern, so that in a comparative estimate the period of incubation, upon which the vitamin concentration in the medium largely depends, needs to be considered. Diagrams B, C and D in Figure 3 represent a different pattern. Here are portrayed the effects of the culture age on the dynamics of vitamin biosynthesis in peptonized milk. In this medium, abundant in organic components, a greatly increased ability to synthesize niacin is found. Its amount rises fivefold or more (pH 6). The synthesizing peak is reached after the 8–10 day period of incubation, after which the biosynthetic ability of vitamin PP becomes lower. A comparison of the maximum values of the rise in levels of niacin in media of different pH values shows an optimum at pH 6.0 or so. Changes in percentages of other vitamins in media are much larger, vitamin B increasing in amount most. Levels of biotin and pantothenic acid definitely drop in the first stage of development and tend to rise towards the completion of incubation. The level of thiamine in media fluctuates and shows two maxima, while that of cobalamin follows an irregular pattern.

(B) Effect of the composition of media on vitamin biosynthetic ability in the strain Penicillium candidum No 2

Comparative tests were carried out on the Capek-Dox media at pH 5.0 and on a 2% solution of hydrolized milk powder at the same pH value. Results are given in Tables 6 and 8 and represented in diagrams A and C in Figure 3. Shapes of the curves emphasize a significant effect of the composition of media on vitamin synthetic abilities in *Penicillium candidum*. In well supplied media the levels of thiamine, riboflavin, pantothenic acid and biotin drop. In view of the dispersion of results the curve depicting the fluctuating level of vitamin B_{12} is of no greater importance.

TABLE 6

CHANGES IN THE CAPEK–DOX MEDIUM
/MEAN VALUES FROM 4 SERIES OF TESTS/

Current No	Incubation period (days)	Total solid of mycelium (g)	Mean volume of medium (ml)	pH	Vitamin B_1 mcg per ml	Vitamin B_2 mcg per ml	Pantothenic acid mcg per ml	Vitamin PP mcg per ml	Biotin mcg per ml	Vitamin B_{12} mcg per ml
1	0	0	0	5.0	66	167	80	275	105	220
2	1		21.6	5.25						
3	2		23.6	5.55						
4	3	0.0272	15.8	6.58	66	148	128	265	180	238
5	4		12.4	6.04						
6	5	0.1306	10.3	4.93	92	272	281		290	313
7	6		12.4	4.98			326	412	325	259
8	7	0.2400	11.6	5.13	126	136				
9	8		10.7	6.00			434	450		332
10	9	0.4400		6.82					590	
11	10		7.9	7.34	142		481	475		
12	11	0.8010		7.66		189				
13	14	1.1059	6.8	8.2	70	359		550	790	290

TABLE 7

CHANGES IN pH (4.5) OF PEPTONIZED MILK
(DIFCO) IN EFFECT OF PENICILLIUM CANDIDUM ACTION

Current No	Incubation period (days)	Total solid of mycelium (g)	Mean volume of medium (ml)	pH	Vitamin B_1 mcg per ml	Vitamin B_2 mcg per ml	Pantothenic acid mcg per ml	Vitamin PP mcg per ml	Biotin mcg per ml	Vitamin B_{12} mcg per ml
1	0	0	0	4.5	455	507	1,235	812	6,750	0.607
2	1		19.7	4.5	405	468	1,220	796	6,700	0.548
3	2		22.1	4.5						
4	3	0.0242	15.9	4.61	470	475	982	800	5,500	0.719
5	4	0.110	15.8	6.33	515	396	750	794	2,325	0.712
6	5	0.1995	20.7	6.41	495	474	730	1,769	3,750	0.871
7	6		17.4	7.27						
8	7	0.2990	7.8	7.78	482	501	625	1,825	2,900	0.700
9	8		1.9	7.39						
10	9	0.3357	9,7	7.05	705	486	782	3,160	3,350	0.593
11	14	0.4237	1.8	7.8	567	753	565	2,481	6,050	

TABLE 8

CHANGES IN pH (5.0) OF PEPTONIZED MILK
IN EFFECT OF PENICILLIUM CANDIDUM ACTION

Current No	Incubation period (days)	Total solid of mycelium (g)	Mean volume of medium (ml)	pH	Vitamin B_1 mcg per ml	Vitamin B_2 mcg per ml	Pantothenic acid mcg per ml	Vitamin PP mcg per ml	Biotin mcg per ml	Vitamin B_{12} mcg per ml
1	0	0		5.0	380	471	1,310	842	732	702
2	1		22.7	5.07			1,220			650
3	2	0.011	22.6	5.01	282	384	1,010	856	602	610
4	3		17.2	6.29			695			615
5	4	0.192	14.4	7.10		365	417	1,625	435	469
6	5		13.9	7.72			460	2,600		444
7	6	0.374	7.2	8.03	247	338	287	3,015	300	765
8	7		6.3	7.9			255		200	690
9	8	0.549	5.6	8.03	200	332	235	4,615	195	841
10	11	0.697	6.1	8.07	247	390	247	4,250	77	771

TABLE 9

CHANGES IN pH (6.0) OF PEPTONIZED MILK
IN EFFECT OF PENICILLIUM CANDIDUM ACTION

Current No	Incubation period (days)	Total solid of mycelium (g)	Mean volume of medium (ml)	pH	Vitamin B_1 mcg per ml	Vitamin B_2 mcg per ml	Pantothenic acid mcg per ml	Vitamin PP mcg per ml	Biotin mcg per ml	Vitamin B_{12} mcg per ml
1	0			6.0	410	490	1,140	837	690	740
2	1		26.4	6.1	283				725	
3	2	0.0615	25.9	6.2	294	488	1,002	785	674	781
4	3		20.6	7.5	315				485	
5	4	0.2301	21.6	7.7	288	495	837	1,567	382	660
6	5		26.1	7.8						
7	6		16.6	8.16						
8	7	0.4899	9.8	8.18	203	558	715	3,417	261	566
9	8		13.1	8.34						
10	10	0.5971	2.7	8.49	294	499	810	5,240	207	
11	12	0.7093	6.2	8.52	298	697	1,230	3,230	727	893

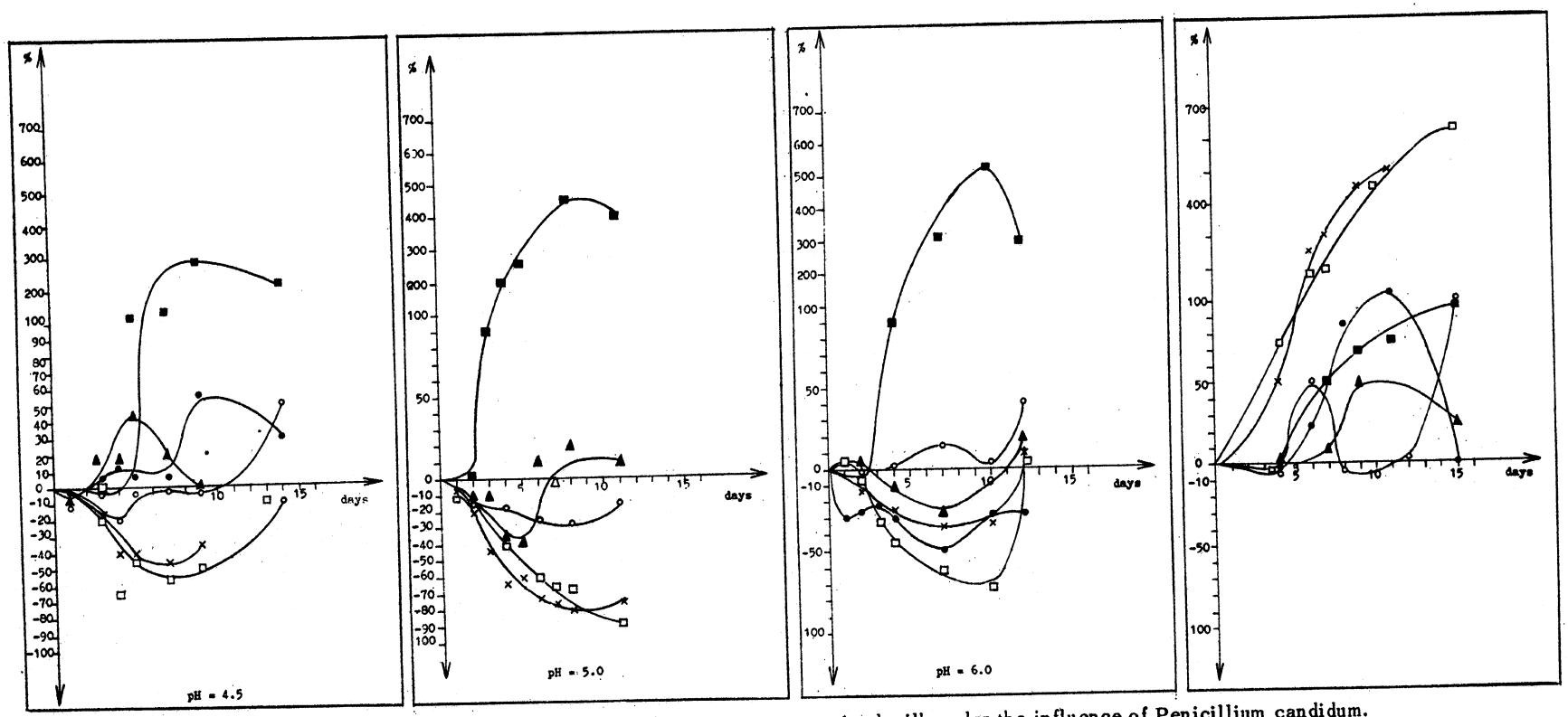


Figure 3. Changes (in per cent) of the vitamin content in peptonized milk under the influence of Penicillium candidum.

(A) at pH = 4.5

(B) at pH = 5.0

(C) at pH = 6.0

(D) Changes of the vitamin content in the Capek–Dox medium (pH = 5.0) under the influence of Penicillium candidum.

Markings:
- thiamine
- niacin
- riboflavin
- biotin
- pantothenic acid
- vitamin B_{12} (cobalamin)

Markings:
- thiamine
- niacin
- riboflavin
- biotin
- pantothenic acid
- vitamin B_{12} (cobalamin)

Markings:
- thiamine
- niacin
- riboflavin
- biotin
- pantothenic acid
- vitamin B_{12} (cobalamin)

Markings:
- thiamine
- niacin
- riboflavin
- biotin
- pantothenic acid
- vitamin B_{12} (cobalamin)

In a simple medium (Capek-Dox) the levels of vitamin B_1, pantothenic acid and biotin continuously rise; increases in the amounts of vitamin B_{12} are negligible. Riboflavin in this medium at first drops to rise rapidly in amount, falls again, and subsequently rises towards the end. The level of niacin behaves differently: in a synthetic medium it rises negligibly, while in hydrolized milk its rise is rapid and conspicuous, only tending to fall during the final stage.

(C) Effect of the medium's pH on vitamin synthetic abilities of Penicillium candidum

Analyses were carried out on 2% solution of peptonized milk at pH 4.5, 5.0 and 6.0, i.e. the Camembert range in which the mold *Penicillium candidum* developes. Results are given in Tables 7, 8 and 9, as well as in diagrams B, C, and D (Fig. 3). They indicate a relationship between the pH and the physiological properties of *Penicillium candidum*. At pH 4.5 the rate of development of the mycelium is much lower than at pH 5.0 or 6.0. The initial acidity bears a marked effect on the following changes in the acidity of medium: in media of pH 6.0 the rise in pH values is at first greater than after a four-day incubation period when it becomes inconspicuous; the maximum occurs after 12 days of incubation. Changes in the acidity of media at pH 4.5 and 5.0 rise to a characteristic peak on the 6–7th day of incubation after which the pH at first falls to rise again eventually. At the present moment an explanation for this peculiar behavior is still lacking.

For the synthesis of thiamine the pH 4.5 of medium is most favorable. Changes in the level of vitamin B_1 are more or less similar at pH 5.0 and 6.0. The biosynthesis of riboflavin is more efficient at pH 4.5 and 6.0 than at pH 5.0. In observing the highest levels of niacin in media of different pH values the optimum in hydrolized milk at pH 6.0 or so is easy to be noticed. For pantothenic acid the optimum pH is 6.0. So it is for biotin, too.

Fluctuations in the amount of cobalamin are insignificant and therefore the optimum acidity cannot be determined.

For most of the vitamins and majority of pH values the increase in vitamin level occurs after a fortnight more or less. The dispersion of results reported by several workers testing the amounts of vitamin B in Camembert cheese may be mainly due to the different ripening periods of the cheese in question.

3. ATTEMPTS TO OBTAIN THE PENICILLIUM CANDIDUM MUTANT

U.V.- IRRADIATION OF MOLD SPORES

The water suspension of spores from the *Penicillium candidum Nos 2* and *10* was irradiated. The density of suspension was standardized to 1.10^7 of spores in 1 ml by a direct microscopical counting in the Thom chamber. The suspension was poured into sterile Petri glasses in volumes allowing to get a 2 mm thick film. The irradiation was carried out with the use of the 30 W Philips quartz lamp propagating its main ray beam with the wavelength equal to 2,537 Å. The distance between the source of wave propagation and the irradiated suspension amounted to 30 cm. The total number of live spores before and after irradiation was determined by inoculation of the liquid into a solid wort-agar medium at pH 4.0. The incubation at 22°C lasted five days.

The mean values obtained from results of five successive irradiations are to be found in Figure 4 depicting lethal properties of spores subjected to irradiation. The irradiation periods ranged from 7½ to 180 minutes. After their irradiation with U.V.- rays the spores were protected from a direct lighting. Their incoulation was carried out immediately after the irradiation.

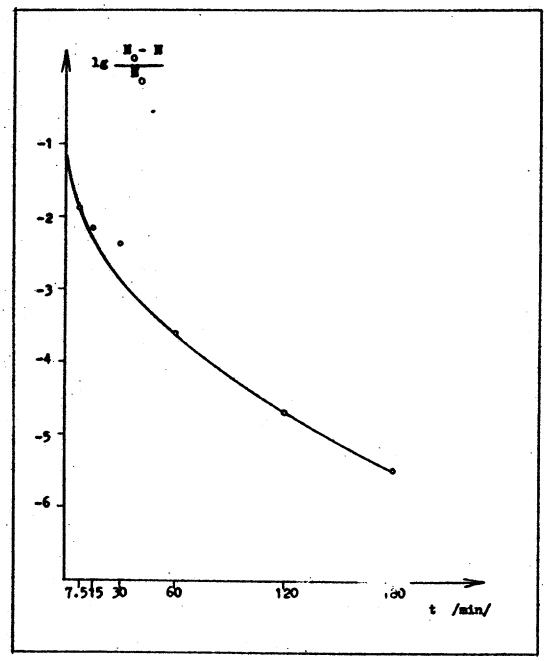


Figure 4. The lethal curve of U.V.-rays action upon the Penicillium candidum spores.

The curve depicting lethal properties of spores is drawn in a half-logarithmic scale and is a second grade curve. From its shape may be seen that already the irradiation for about 8 min. resulted in destruction of about 99.9 per cent of spores. Thus, for isolation of mutants the 10-min. periods of irradiation have been accepted.

For a criterion of the primary isolation of populations characteristic of their better abilities to produce vitamins an ascertainment was adopted that a strain being auxotrophic with regard to a given vitamin is able to develop in a medium lacking this growth factor. Further on, it has also

been accepted that the rate of growth in such a vitaminless medium may be regarded as a measure of abilities of a given population for biosynthesis. In this connection the spore suspension after the 10-min. irradiation was appropriately dissolved and then inoculated to the Capek-Dox medium with an agar addition at pH 4.0. From the colonies grown in a medium containing merely the traces of vitamin only the biggest were isolated. The selected mutants were in turn evaluated as to their growth rate in the liquid Capek-Dox medium containing all vitamins excluding one of them. Data relating to such an isolation given in Table 10 should be regarded merely as examples.

Results in Table 10 represent the mean weights of a dried mycelium grown in a medium after a 3-week incubation period. These trials may be regarded as a further step in selection of mutants. From among the represented mutants those numbered 10–A and 10–C were characteristic of their poorest development, whereas 10–D, 10–H and 10–E were developing considerably faster. The same system was applied further for a primary isolation of mutants obtained in a further series of trials.

didum strain *No 2*. Here a lower rate of development of mutant molds in comparison with the parent strain should be reported. In order to obtain a normal degree of ripeness the cheeses needed to be ripened a week longer than normally. It seems probable that a bitter taste occuring frequently enough was due to the products of mold autolysis.

The velocity of germination and that of growth of the mold mutants on a broth substrate with agar, and on the Capek-Dox agar medium, is on the average smaller than in the parent strains. Yet, individual mutants differ greatly as to their germination rates. This property was estimated under the microscope. The percentage of germinated spores incubated at 25°C for 18 hours in broth is represented by the following average values:

10–A – 50.9%	10–L – 47.6%	2–A – 83.6%
10–B – 23.6%	10–M – 65.0%	2–B – 92.0%
10–C – 42.7%	10–S – 44.7%	2–C – 54.3%
10–D – 46.2%	10–H – 44.7%	2–D – 62.8%
10–E – 4.5%	10–K – 39.8%	2–E – 53.6%
10–F – 52.1%	10–Z – 46.2%	10–F – 85.0%
	average = 42.1%	average = 71.9%

TABLE 10

GROWTH RATE OF MYCELIUM FROM SEVERAL ISOLATED MUTANTS OF PENICILLIUM CANDIDUM GROWN IN MEDIUM LACKING PARTICULAR VITAMINS

Mutant	Mean weight of mycelium (g) after the 3-week incubation on media in which the following vitamins were lacking respectively						
	0	B_1	B_2	Pantothenic acid	H	B_6	Paraamino-benzoic acid
10–A	0.0926	0.2505	0.2178	0.0235	0.2494	0.2678	0.2701
10–D	0.4718	0.5192	0.5075	0.5534	0.4287	0.3439	1.7685
10–C	0.1407	0.1317	0.1075	0.1890	0.0986	0.0813	0.1456
10–E	0.3865	0.3783	0.3818	0.4377	0.4671	0.5801	0.4418
10–H	0.4037	0.4761	0.4375	0.4204	0.4273	0.5484	0.4786

(A) ISOLATION AND APPRAISAL OF THE PENICILLIUM CANDIDUM MUTANTS

Mutants isolated after the U.V. and X-ray irradiation of *Penicillium candidum* strains *Nos 2* and *10* were submitted to a technological evaluation. Three series of Camembert cheese manufactures were carried out. Cheeses belonging to the same series were sprayed with a spore suspension (10^6 per 1 ml) of different mutants. The spores originating from the 3-week old populations cultured on the wort-agar cheeses ripened for 4 weeks and were then submitted to sensory tests by a three-person panel of experts.

The results of sensory tests emphasize an improved quality of cheeses manufactured with the use of mutants derived from the *Penicillium candidum*

In order to induce mutants with qualities differing more largely from that of the parent strains a few mutants were X-rayed for a second time. Doses applied were 200,000 and 135,000 r.

Spores of individual mutants possess a different resistance to X-rays. It has been found that the mutant 10–K was most susceptible, while 10–X most resistant. The differences in the velocity of growth of mutants deserve to be emphasized. The diameters of stationary cultivated colonies may be regarded as an indication of the rate of mold development. Analyses of samples revealed a retarded growth of irradiated spores, evident from a higher percentage of smaller colonies in comparison with the parent strain. These findings are in accordance to previously made observations of a reduced rate of mold development on the Capek-Dox medium.

The arrested development of molds in vitaminless media after irradiation may be explained by their limited ability to biosynthesize vitamins.

X-ray irradiation

A set of Rontgen lamps of 220 kV, 24 mA was used for irradiations. Samples were irradiated in test tubes of 1 ml ± 0.1 ml and to each tube an identical control tube with chemical dosimeter was added.

The required range of the total irradiation dosis was obtained within the time of ½ to 3 hours. The dosimetric method of Weiss, normally applied to measure the gamma-ray dosis was used to define the dosis of X-rays. This method is based on determination of concentration of ferric ions formed from the ferrous ions under the influence of X-rays. The dosis of 1,000 r is able to oxidize 16 M Fe. The ferric ions content was determined by the colorimetric method at which the colored reaction with ammonium rhodanide was applied. Three series of irradiations with X-rays of *Penicillium candidum No 2* and *No 10* were carried out. The irradiations in a particular series were performed in doses fluctuating from a few thousands to about 60,000 r per 1 ml of spore suspension. The results concerning the lethal effect of irradiation are presented in Figure 5.

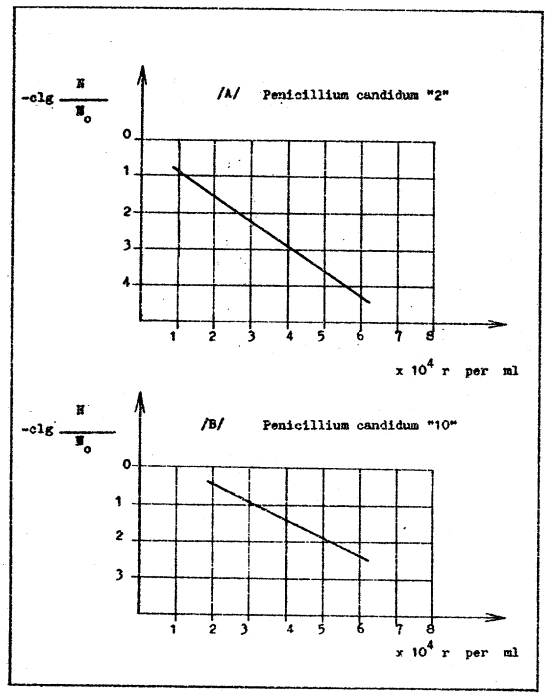


Figure 5. The lethal effect of X-rays irradiation on the Penicillium candidum spores.
(A) Penicillium candidum No 2
(B) Penicillium candidum No 10

The strain No 10 has proved to be much more resistant against the X-ray action than the strain No 2. The differences between the above strains are distinctly recognizable in Figure 5. Mutants were selected by U.V.- irradiation in the same manner as described above.

Gamma-ray irradiation

The spore suspension in saline was irradiated in test tubes of ca 1 ml volume by means of ^{60}Co source of gamma-rays on a stand for standardized test tubes. The doses, corresponding to the following distances from the gamma-ray source were:

distance in cm	dosis in r per hour
2.5	44,500
3.5	27,000
4.5	17,000
5.5	12,000

A series of irradiations was performed for each strain with the varying of dosis by an adjustment of the distance between the source of ray propagation and the irradiated suspension, as well as the time of exposure. The doses ranged from 1,000 to 600,000 r per hour.

It has been established in the course of preliminary experiments that the lethal effect of irradiation on the spores could only be recognized after an application of doses exceeding 120,000 r per hour. The lethal effect of gamma-rays is shown in Figure 6.

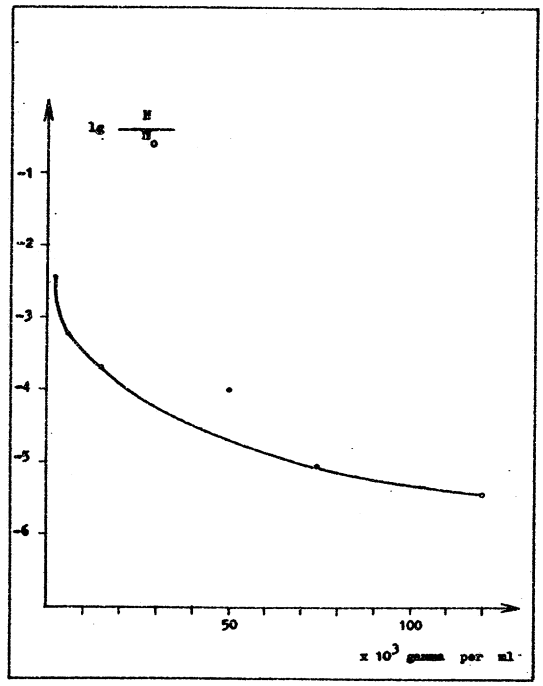


Figure 6. The lethal curve of gamma-rays action upon the Penicillium candidum "2" spores.

(A) Mutants of Penicillium candidum

Forty-five mutants of *Penicillium candidum Nos 2* and *10* were isolated in total. As the next isola-

tion step a technological evaluation of mutants in the Camembert cheese manufacture was adopted. Three series of cheese manufactures (A, B, C) with different water content were carried out. From among them cheeses belonging to the series "A" were most hard, whereas those belonging to series "B" had normal consistency and the cheeses of the series "C" were soft. Such differentiation of cheese structure permitted to estimate the mold quality in different conditions. These cheeses after draining off were sprayed with the suspension of mutant spores (after 1-month cultivation) and the next day salted dry. After the 4-week ripening period they were submitted to sensory test by a five-person panel of experts. In accordance with regulations in force in Poland the cheeses were classified into three main categories: 1, 2 and 3 and besides into three respective sub-categories: 1/1, 1/2, 1/3; 2/1, 2/2, 2/3; 3/1, 3/2, 3/3. From among 45 mutants five have been chosen, namely Nos 6, 10, 17, 45 and 47, securing very good quality of cheese, as well as four other mutants with specific, distinct properties. The mutants Nos 20, 35, and 28 were characteristic of their strong lipolytic properties causing that the taste of cheese made with the use of these mutants was resembling rather that of Roquefort not of Camembert. The mutant No 38 distinguished itself with strong proteolytic properties. Cheeses manufactured with the use of mutants Nos 6, 10, 17, 45 and 47 were subjected to a more detailed chemical analysis and also to tests purposed to detect the B-group vitamins in them.

CHARACTERISTICS OF MORPHOLOGICAL FEATURES (APPEARANCE OF COLONIES OF SELECTED MUTANTS OF PENICILLIUM CANDIDUM)

The colonies of mutants after a 7-week cultivation period in the Capek-Dox medium had the following appearances:

No 6 (U.V.-10 – 11) – flat, round, regular in shape, white-colored colonies with slightly uprised, pointed center; diameter of the colony – 2.0 cm;

No 10 (U.V.-10 – 2) – white-colored colonies, surrounded by the radially spreading shreds with slightly uprised center and a dense mycelium; average diameter of the colony – about 1.0 cm;

No 17 (U,V,- 2-F) – white-colored, round colony with a slightly folded bottom and a loose mycelium; diameter of the colony – about 1.4 cm;

No 45 (X – 21) – white-colored, round, uniformly flat colony with very short shreds; average diameter of the colony – about 1.5 cm;

No 47 (X – 25) – white-colored, round colony with a slightly uprised center around which the regular hollows are visible surrounding elevation; edges slightly uprised; average diameter of the colony – 2.0 cm;

No 38 (X – 10/IV) – slightly pale-colored colony with characteristic concentric folds on the surface of mycelium; drops of liquid are visible after a longer period of cultivation; average diameter of the colony – 2.5 cm;

No 28 (gamma – 201) – white-colored, round colony with a dense, close mycelium; average diameter of the colony – 1.8 cm;

No 35 (X – 10-8) – a colony by its very characteristic appearance greatly differing from other colonies (cf. Figure 7).

This colony is pale-colored, with distinct mycelium shreds around it and has a slightly uprised center and a skin-like mycelium. After a longer cultivation in the centers of some older colonies appear the irregularly shaped holes reaching the surface of mycelium; average diameter of the colony – about 0.6 cm;

No 20 (gamma – 10-3) – white-colored, flat colony with the slight concentric folds; diameter of the colony – about 1.5 cm;

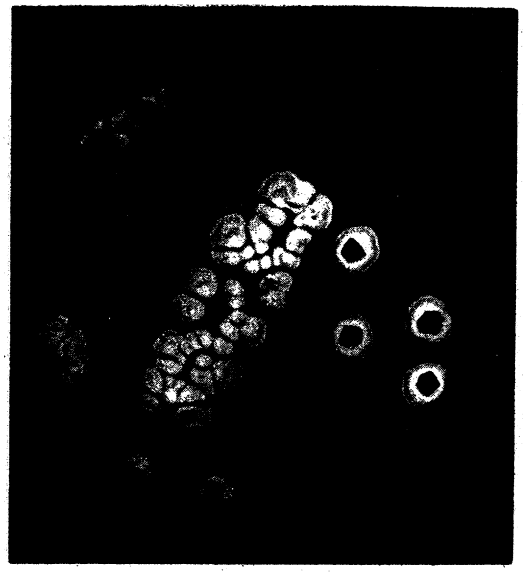


Figure 7. A specific appearance of a colony of mutant No 35 (X–10–8) of Penicillium candidum.

The chosen mutants were subjected to further investigations concerning their abilities to synthesize the B-group vitamins in Camembert cheese, as well as to those with the aim to clear up their lipo- and proteolytic properties.

BIOCHEMICAL EVALUATION OF SELECTED MUTANTS

The proteolytic properties of these strains have been characterized as well as their abilities to biosynthesize the B-group vitamins. Investigations were performed on cheese as the culture medium

and at the same time the rate of protein changes in effect of selected mutants action and the vitamin content were investigated.

In order to examine the durability of properties in chosen mutants after a 1-year preservation of their lyophilized spores the repeated manufactures

TABLE 11

PROTEOLYTIC PROPERTIES OF SELECTED MUTANTS OF PENICILLIUM CANDIDUM

Strain No	Protein in cheese %	Soluble protein %	Soluble protein in % proportion to total protein
Parent strain No 2	21.39	5.66	26.46
No 6 (UV 10–11)	21.84	6.71	30.72
No 10 (UV 10– 2)	21.15	8.74	41.32
No 17 (UV – 2F)	21.47	4.91	22.87
No 45 (X – 21)	21.26	5.03	23.65
No 47 (X – 25)	21.95	6.29	28.65

The proteolytic changes were evaluated by determing the percentage of soluble protein (in effect of precipitation from the water emulsion with HCl at pH 4.5) in proportion to the total protein content by the Kjeldahl method (see data in Table 11). It has been shown experimentally that the *Penicillium candidum* mutant No 10 can be regarded as one possessing best proteolytic properties. In some cases the soluble protein content in cheese exceeded by about 2 times that in cheese manufactured with the use of the parent mold strain. A fairly apparent increase in content of soluble products of protein hydrolisis has been observed in the *Penicillium candidum* mutant No 6 and in some extent in the mutant No 47.

of cheeses were performed.

Six series of experimental manufactures of Camembert cheese have been carried out in the same manner as in the course of previous experiments. The parent strain of *Penicillium candidum* No 2 and the *Penicillium candidum* mutants Nos 45 ("X–21") and 47 ("X–25") have been taken for evaluation. The mean values are presented in Table 13 (part A).

From the values obtained may be seen that the *Penicillium candidum* mutant No 45 distinguished itself by its higher ability to biosynthesize vitamin B_2, whereas the ability to produce vitamins shown by the mutant No 47 was lower if compared

TABLE 12

EVALUATION OF THE PENICILLIUM CANDIDUM MUTANTS AS TO THEIR ABILITY TO BIOSYNTHESIZE VITAMINS SELECTED IN EFFECT OF TECHNOLOGICAL ASSAY

Strain No	Vitamin B_1 mcg per 100 g cheese	Vitamin B_2 mcg per 100 g cheese	Vitamin PP mcg per 100 g cheese	Pant. acid mcg per 100 g cheese	Biotin mcg per 100 g cheese	Vitamin $_{12}$ mcg per 100 g cheese
Parent strain No 2	62.3	610	796	257	8.15	0.16
No 6 (UV–10–11)	62.5	557	760	186	6.37	0.11
No 10 (UV–10–2)	65.3	573	943	373	5.27	0.15
No 17 (UV – 2F)	69.9	540	680	188	3.65	0.12
No 45 (X – 21)	69.2	624	720	142	5.65	0.14
No 47 (X – 25)	62.4	549	986	327	4.68	0.13

The ability of the *Penicillium candidum* mutant No 47 to biosynthesize niacin and pantothenic acid has proved to be higher respectively by about 25% and over 20% than that of the parent strain. The mutant No 45, however, is able to produce quite insignificantly more vitamin B_1 when compared with its parent strain. Besides the *Penicillium candidum* mutant No 10 has secured by about 20% higher average content of vitamin PP in cheese and also a stronger concentration of pantothenic acid (by about 45%). In view of their technological properties and the ability to produce vitamins the mutants Nos 45 and 47 have been chosen as most suitable ones.

with that of its parent strain.

On the assumption that a general decreasing of the ability to biosynthesize vitamins by the strains under investigation might be regarded as a result of a partial weakening of their vitality by preservation of lyophilized spores it has been decided to make a series of their inoculations into the agar-wort medium and then to subject them to evaluation.

Two series of cheese manufactures were carried out and their results are presented in Table 13 (part B).

In the course of tests mutant No 45 has distinguished itself by the ability to synthesize vitamin

TABLE 13

EVALUATION OF THE PENICILLIUM CANDIDUM MUTANTS AS TO THEIR ABILITY TO BIOSYNTHESIZE VITAMINS

Strain No	Vitamin B_1 mcg per 100 g cheese	Vitamin B_2 mcg per 100 g cheese	Vitamin PP mcg per 100 g cheese	Pant. acid mcg per 100 g cheese	Biotin mcg per 100 g cheese	Vitamin B_{12} mcg per 100 g cheese
	A. Mean values from the I series of cheese manufacture					
Parent strain No 2	154	802	1,331	608	5.58	1.37
Mutant No 45	152	847	1,365	547	7.33	1.28
Mutant No 47	134	773	1,328	560	5.72	1.34
	B. Mean values from the II series of cheese manufacture					
Parent strain No. 2	141	558	1,428	300	3.38	2.29
Mutant No 45	145	538	1,835	400	2.63	2.49
Mutant No 47	188	531	1,260	275	3.3	2.02

PP and pantothenic acid, whereas the mutant No 47 produced greater amounts of thiamine than the remaining strains.

During the second series of tests the lypolitic changes were analysed and the following results were obtained:

acid number in cheese with *P.candidum* strain No 2 – 7.9.

acid number in cheese with *P.candidum* mutant No 45 – 7.2.

acid number in cheese with *P.candidum* mutant No 47 – 8.9.

The above interrelationship has also been confirmed by testing the fat isolated from samples and heated at 105°C for 5 hours. Here the results were as follows:

for the sample No 1 – 18.5.

for the sample No 2 – 19.2.

for the sample No 3 – 24.2.

Neither in the course of peroxide, nor the TBA-tests any values have been found. It should be emphasized that the cheeses manufactured with the use of the above-mentioned mutants have gained the best scores with respect to their taste in the course of sensory tests.

4. CHARACTERISTICS OF TECHNOLOGICAL UTILITY AND ESTIMATION OF BIOCHEMICAL PROPERTIES OF VARIOUS PENICILLIUM ROQUEFORTI STRAINS

The biochemical properties of seven *Penicillium roqueforti* strains have been characterized by their direct action in cheese.

Four series of experimental manufactures of Roquefort-type cheese from the cow milk, using the same milk and applying the same production conditions, were carried out.

The cheese grain was sprayed with the spore suspension of molds under investigation. After a 3-month ripening in a cellar at 8 – 10°C and humidity amounting to 90 – 95 per cent the cheeses were subjected to sensory tests by a panel of experts and also to a laboratory examination. The cheeses were assayed according to regulation in force in Poland providing that the characteristics may be qualified as the first, second or third class with a further differentiation into subclasses. Accordingly the score "2/3" denotes that in the assay of a given property the cheese was qualified as belonging to the second class and within it to the subclass 3. In Table 14 are presented the results of the cheese taste assay. Characteristics somewhat differing from those standard, considered as good for Roquefort cheese, are besides described in words and provided with the numeric qualification.

The results of this assay indicate that the cheeses manufactured with the use of the same strain also differ to some extent, thus pointing to the influence of manufacturing conditions. The susceptibility of the investigated strains to external conditions was observed to be varying. A conventional scoring applied according to Table 14 and the calculation of standard deviations of individual results from the mean values allowed to establish that the strain *Penicillium roqueforti No 5* exhibits stable features, while the strains Nos 3 and 6 show slight variations only.

According to a qualitative score the strains may be arranged in the following order: the best cheeses were obtained with the *Penicillium roqueforti* strain *No 3*, as the next came Nos 2 and 5, on the whole providing a satisfactory stability of their characteristics.

However, the different strains gave the growth of unequal rate in cheeses. In order to express this quantitatively the Gerber photocolorimeter was used in the following manner: a segment of cheese was ground in a mortar and the bluish mass was then placed in a cylindrical vessel. The surface of cheese was covered with a piece of glass and the color intensity measured by means of the Gerber Z 4800 electrophotometer normally applied for determination of the degree of milk contamination or examination of the butter and milk coloring.

TABLE 14

RESULTS OF A TASTE ASSAY

Penicillium roqueforti strain No	Series I	Assay	Series II	Assay	Series III	Assay	Series IV	Assay	Total score *)	Mean assay
1	slightly stinging slightly soapy	2/3	pure tyrosine flavor less strong	1/2	musty, stinging Limbourg flavor	2/1	pleasant mushroom flavor slightly bitter, piq.	1/3	15	2/1
2	indefinite hardly typical	2/1	slightly stinging somewhat rancid	1/2	mody flavor too strong	1/3	pleasant piquant	1/2	11	1/3
3	slightly stinging hardly typical	2/1	strong pure slightly stinging somewhat rancid	1/2	piquant typical	1/3	typical piquant slightly rancid	1/1	10	1/2--1/3
4	slightly rancid slightly soapy	2/1	pure slightly stinging	1/3	stinging very strong	2/2	typical; piquant; slightly rancid; somewhat bitter;	1/2	14	1/3--2/1
5	slightly sour very slightly stinging	1/3	slightly sour somewhat rancid less stinging	1/3	piquant rather strong slightly rancid	1/3	somewhat stinging	1/3	12	1/3
6	slightly sour non-typical	2/1	sour indefinite curd-like	2/2	strong moldy, unpleasant taste	2/1	slightly piquant Limbourg flavor	1/3	16	2/1
7	indefinite slightly acidulous slightly piquant	1/2	indefinite curd-like sour non-typical	2/1	strong somewhat bitter slightly rancid	2/1	soapy stinging bitter	2/2	15	2/1

*) The following criteria were adopted in scoring: assay 1/1 – score 1; 1/2 – score 2; 1/3 – score 3; 2/1 – score 4; 2/2 – score 5; 2/3 – score 6.

TABLE 15

PENICILLIUM ROQUEFORTI MOLD GROWTH IN CHEESE

Strain No	Series I	Coefficient *)	Series II	Coefficient	Series III	Coefficient	Series IV	Coefficient
1	too little mold in site of infection	3.4	slightly not uniform	6	too exuberant	1	not quite uniform	7
2	uniformly distributed	1	uniform	1.7	too exuberant	1.7	exuberant uniform	2.3
3	too little mold	5	uniform slightly irregular	2.3	a little too weak not uniform	4.5	uniform	1.7
4	too little mold	2.8	uniform slightly irregular	6	not uniform	3.1	not uniform	2.5
5	weak	7	somewhat too weak	2.7	not uniform	2.5	somewhat too weak	5.1
6	too little mold	3.2	very weak	7	completely uniform	1.8	not quite uniform	3.4
7	regular	2.1	uniform	3.1	not quite uniform	2.2	somewhat too weak	4.8

*) The coefficient represents a value by which the figure for mold growth should be multiplied in order to obtain the growth rate equal to that in the optimum sample (i.e. Series III, No 1)

The compensation of the light intensity from a standard bulb and of reflected light was achieved by turning the left regulating knob labelled "Unfiltriert" (not filtered) and then the shift was read from a scale.

Tracing of a standard curve, To determine the rate of mold growth in cheese the color intensity of the cheese mass colored most strongly was conventionally accepted as 1. This mass was successively mixed with the increasing portions of a moldless cheese and the scale was read. Results were traced in Figure 7, referring to the X-axis, the "degree" of dissolution of the standard-colored cheese mass with moldless cheese addition, and to the X-axis readings of the scale. The coefficients plotted on the X-axis represent the reciprocal of the mold growth rate in relation to the standard cheese. The results of both visual and colorimetric estimation are given in Table 15.

As it may be seen from data in the above Table the *Penicillium roqueforti* strain *No 2* secures a uniform, rather intensive growth which is almost equal in all series of manufactures. As far as these characteristics may be concerned the strain No 7 has proved to be of somewhat inferior quality.

to yellow. The small differences in the dry weight of cheeses obtained from separate manufacture series (Table 17) provide an evidence that the conditions of experimental manufacture were constant enough. However, a higher average quality of cheeses from the second and fourth series is connected with a lower dry weight, amounting to about 60 per cent. This observation does not, however, permit any generalization and only suggests a possibility that some relation exists between the quality of cheese and its dry solid. In the cheese compared no wider differences have been found in pH. The mean values of pH lie within the limits 5.25 – 5.60.

From among the strains of *Penicillium roqueforti* under investigation Nos 3, 5 and 7 possess somewhat weaker proteolytic properties, while Nos 6 and 2 are stronger (see, Table 18). A general statement may be made that the differences in the percentual amine nitrogen content as compared with a total N content in cheeses manufactured with the use of different *Penicillium roqueforti* strains are but slight.

The lipolytic properties are presented in Table 19. The cheeses with a high score from sensory tests

TABLE 16

COLOR OF MOLDS IN ROQUEFORT–TYPE CHEESE

Penicillium roqueforti strain No	Series I	Series II	Series III	Series IV
1	green	yellowish green	blue-grey with yellowish shade	blue with greenish and yellowish shade
2	dark green	grey-green	blue with grey-green shade	grey-green-blue
3	grey-green	grey-blue, here and there yellow-green	blue and grey (two-colored)	grey-green-blue
4	dark grey--green	green-grey with yellow hue	blue and dark (two-colored)	grey-blue
5	bluish (nice color)	green-grey with yellow hue	blue with greenish hue	greenish-blue
6	yellow-green (two-colored)	light grey	blue with greenish hue	green and blue (two-colored)
7	blue-grey (nice color)	green and blue (two-colored)	blue with greenish hue	green and blue (two-colored)

The mold in cheese shows a smaller or greater tendency to change its color to a yellow hue, sometimes passing even to brown. When the cheese is cut, the access of oxygen causes this color to change once more to blue with a greyish or greenish shade. The results of the color estimation of the mold grown in cheese are given in Table 16. The strains Nos 3 and 5 show normal color in successive products, while No 1 tends to change

contain fat but little decomposed. Acidity of fat in cheeses manufactured with strains Nos 2, 3 and 5 is lowest, the smallest dispersion of results being found for the cheeses with *Penicillium roqueforti No 5*, and successively Nos 3 and 2. Wide variations and the high fat acidity values are observed for strain No 6.

The majority of strains with a weak hydrolytic activity exhibit a stronger oxidative activity. As

TABLE 17

SOLID CONTENT IN PER CENTS

Penicillium roqueforti strain No	Series I	Series II	Series III	Series IV
1	62.44	61.81	–	61.63
2	60.48	61.12	61.51	60.75
3	63.00	60.42	60.75	59.95
4	63.30	62.20	61.08	58.02
5	62.76	58.10	59.41	58.51
6	62.17	58.06	59.05	60.78
7	63.13	60.06	61.39	59.26
Mean	62.47	60.25	60.65	59.84

TABLE 18

PROTEOLYTIC ACTIVITY OF PENICILLIUM ROQUEFORTI STRAINS

Penicillium roqueforti strain No	Nitrogen per cent in solid				Amino nitrogen as per cent of total nitrogen		
	S	e	r	i	e	s	
	I	II	III	IV	II	III	IV
1	6.33	5.91	6.02	5.75	–	30	30
2	6.04	6.74	6.13	5.86	33	32	36
3	6.40	5.91	6.93	5.82	29	27	31
4	5.85	6.64	5.70	5.88	30	34	29
5	5.20	6.75	5.72	6.12	–	29	28
6	6.53	6.26	6.01	6102	32	32	36
7	6.11	6.54	5.75	5.96	23.5	35	25

TABLE 19

LIPOLYTIC ACTIVITY OF PENICILLIUM ROQUEFORTI STRAINS

Penicillium roqueforti strain No	Acidity of cheese fat				Peroxide number of cheese lipids			
	S		e	r	i		e	s
	I	II	III	IV	I	II	III	IV
1	16.5	46.7	19.7	13.2	0	0	1.0	2.5
2	32.1	29.9	11.2	17.8	0	1.27	1.6	2.0
3	21.7	9.9	33.3	18.1	1.6	1.0	5.6	0
4	28.9	32.6	55.7	45.2	0	2.0	0.44	2.42
5	24.1	24.2	31.6	13.3	0	0	0.68	1.58
6	5.8	52.5	74.5	53.9	0	0.6	1.12	1.06
7	7.4	32.6	48.4	36.1	0	1.2	0	0.8

an exception here may be quoted the strain No 5 with its weak lipolytic and oxidative activity. The thiobarbituric acid (TBA) test did not reveal any further fat oxidation products in cheese. The results of analysis for vitamin B content in experimental cheeses indicate a wide dispersion of their values obtained for a given strain in the successive manufacture series.

Significant fluctuations occur in the biotin content between the cheeses from particular manufacture series while using the same strain. In some cases differences are amounting to tenfold values. This points to the essential role of the rate of natural medium, i.e. cheese, in biosynthesis of biotin on which the strains of *Penicillium roqueforti* are developing. This problem, however, seems to require further investigations in the future. The contents of remaining vitamins did not show such great differences.

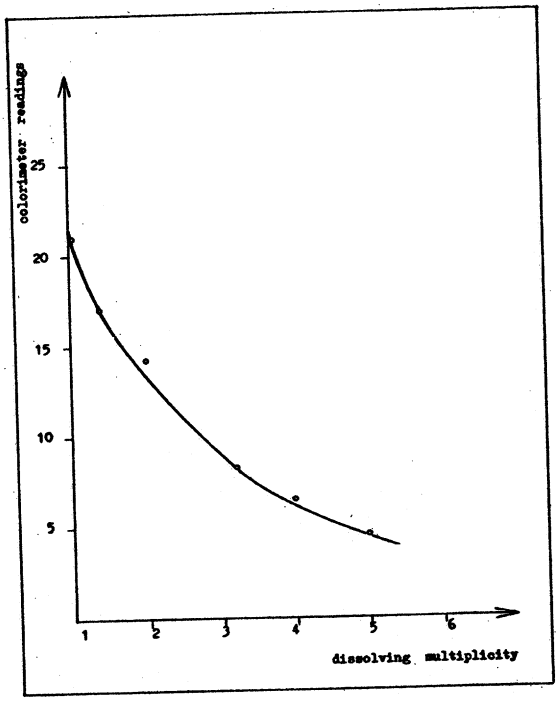


Figure 8. A standard curve. Relation between dissolving multiplicity of standard cheese mass and Gerber's colorimeter readings.

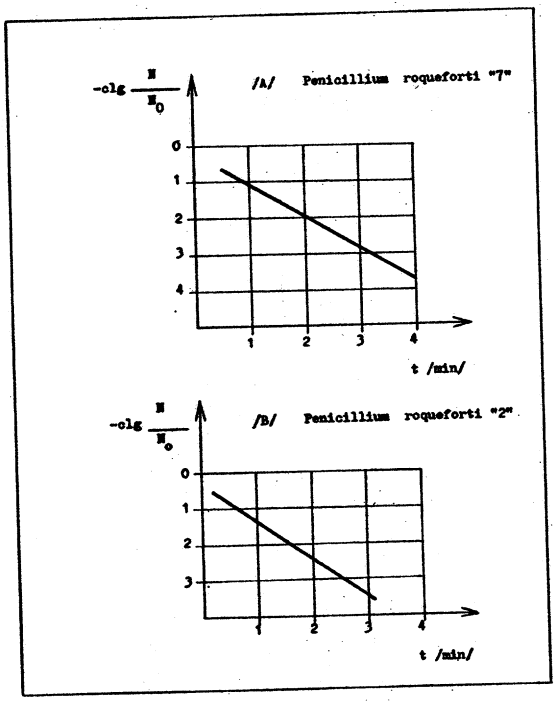


Figure 9. The lethal effect of U.V.-ray irradiation on the Penicillium roqueforti spores.
(A) Penicillium roqueforti No 7
(B) Penicillium roqueforti No 2

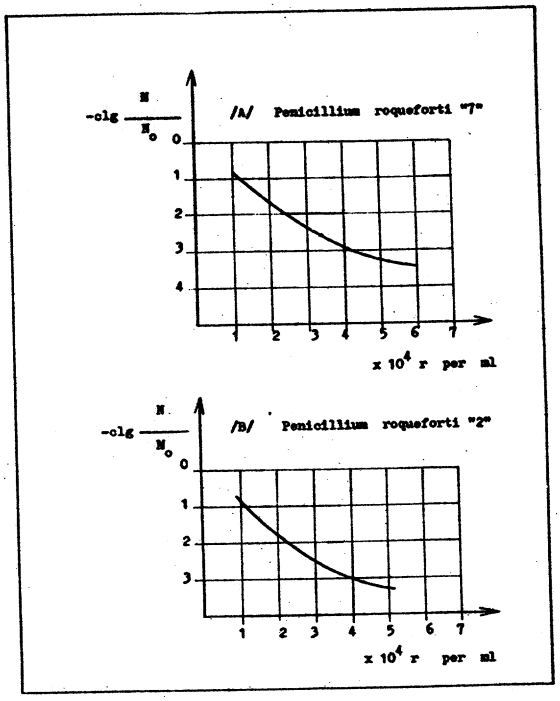


Figure 10. The lethal action of X-rays upon the Penicillium roqueforti spores.
(A) Penicillium roqueforti No 7
(B) Penicillium roqueforti No 2

5. ATTEMPTS TO OBTAIN THE PENICILLIUM ROQUEFORTI MUTANTS

The *Penicillium roqueforti* strains *Nos 2* and *7* selected in effect of evaluation of their technological utility and their abilities for biosynthesis were subjected to action of mutagenic factors, namely they were irradiated with U.V.- and X-rays. The conditions in which the suspension of spores has been prepared and the irradiation performed were identical with those during the attempts aimed to obtaining the mutants of *Penicillium candidum.*

From an irradiated suspension of spores the quantitative dilutions of 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 were prepared and from each one of them inoculations were made to the wort-agar medium and to the Capek-Dox medium with an agar addition.

A comparative evaluation of the quantity of colonies obtained from the initial spore suspension and of those subjected to irradiation was taken as a basis for a further appraisal of the lethal effect of irradiation.

To isolate the mutants distinguishing themselves by their greater ability to biosynthesize the B-group vitamins the spores after their irradiation were inoculated on the vitaminless Capek-Dox medium with agar addition, from which the greatest colonies have been selected for further investigations, especially these with their diameter exceeding those of the parent strain. Besides of biochemical mutants some quantity of specific morphological mutants has also been selected.

LETHAL EFFECT OF U.V.- IRRADIATION

The procedure of irradiations with U.V.-rays was the same as described under the paragraph "U.V.-irradiation of mold spores" (see, page 17), the aqueous suspension of spores being exposed to the rays action for 15 and 30 sec or 1, 2, 3 and 4 minutes. The effectiveness of irradiation was expressed as a co-logarithm of the ratio of quantity of spores after irradiation to that before the irradiation. The mean values calculated from the three parallel tests were taken as a basis for tracing the curves depicting the lethal effect of U.V.-rays on the *Penicillium roqueforti* strains *Nos 2* and *7* (see, Figure 8). It has been found that the lethal action of rays may be assumed as proportional to the irradiation time. It has been also observed that the strain *Penicillium roqueforti No 7* is slightly more resistant to U.V.-rays than the strain No 2.

LETHAL EFFECT OF X-IRRADIATION

Three series of irradiations of the *Penicillium roqueforti Nos 2* and *7* spores were carried out. Between the results of particular series considerable differences have been notice probably connected with the fact that the spores of *Penicillium roqueforti* in water suspension possessed an insufficient durability. On the other hand this suspension had proved not uniform enough because of tendency of the spores themselves to rise to the surface of liquid. This phenomenon results in reduction of an effective repeatableness of consequences of the lethal action of X-rays and in addition is negatively influencing the accuracy of the spore quantity by the plate method. Neverthelles, the mean values from the three series of tests permited to trace the curve depicting the lethal effect of X-rays (Figure 9) in a satisfactory manner.

As it was the case with the U.V.- irradiation the *Penicillium roqueforti* strain *No 7* has proved more resistant to the X-ray action. A destruction of about 99 per cent of spores can be achieved at the ray dosis within the limits from $2.5.10^4$ to $3.0.10^4$ r per 1 ml.

6. EVALUATION OF MUTANTS OBTAINED FROM THE PENICILLIUM ROQUEFORTI STRAINS Nos 2 AND 7

Five mutants from the *Penicillium roqueforti No 2* and the same number from *Penicillium roqueforti No 7* have been isolated in total. Their characteristics as to the technological utility were based upon the evaluation of organoleptic qualities of the cheese mass ripened under the action of isolated mutants. The same samples have been used for characterizing the biochemical properties of the mutants under investigation. To avoid differentiation of the quality of cheese obtained under the action of the same molds, caused by an unequal growth of mycelium in the inner parts of cheese, these investigations have been modified as follows: from the pasteurized cow milk the cheese was prepared in the manner applied in manufacturing of Blue cheese variety, however, without the mold spores addition. After allowing it to drain and salting, the cheese has been cut into 2.5 cm cubes which were placed in the Roux bottle and then inoculated with abundant amounts of mold spores from the mutants under investigation.

After a two-week ripening period at 15°C the well-ripened cubes, covered with the fully-grown mycelium were taken for sensory tests and one part of them rubbed up in mortar and conveyed to further investigations. The total protein content, soluble protein content, fat acidity, peroxide number, TBA (thiobarbituric acid) index and the B_1, B_2, PP vitamin, pantothenic acid, biotin and the B_{12} vitamin content determinations were performed by the use of the methods described in the early parts concerning the *Penicillium candidum* (see, pages 5–7).

Three series of experimental manufactures of cheese were carried out and the results from chemical analyses of the cheese cubes after a 2-week ripening period are presented in Table 20.

As it may be seen from experimental results the *Penicillium roqueforti* mutant "7/4" exhibits

TABLE 20

B–GROUP VITAMINS CONTENT IN ROQUEFORT CHEESE PRODUCED BY USE OF DIFFERENT PENICILLIUM ROQUEFORTI STRAINS

Strain No	B_1			B_2			PP			Pantothenic acid			Biotin			B_{12}		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	220	30	1.0	1050	610	2.8	440	200	1.8	630	260	1.0	5.31	2.21	2.1	2.27	0.87	5.1
2	220	30	1.0	1010	570	2.5	710	470	4.2	710	340	1.3	4.30	1.20	1.1	2.87	1.47	8.6
3	180	10	–	790	350	1.5	375	135	1.2	740	370	1.4	4.13	1.03	1.0	1.98	0.58	3.4
4	250	60	2.0	740	300	1.3	650	410	3.7	780	410	1.5	11.75	8.67	8.4	1.84	0.44	2.5
5	250	70	2.3	700	260	1.1	360	120	1.1	730	360	1.3	5.71	2.61	2.5	2.06	0.66	3.8
6	270	80	2.6	660	220	1.0	580	340	3.1	870	500	1.9	12.10	9.00	8.7	1.57	0.17	1.0
7	260	70	2.3	750	310	1.2	350	110	1.0	735	365	1.4	7.67	4.57	4.4	1.66	0.22	1.3
Control (raw cheese)	190			440			240			370			3.10			1.40		

a – Vitamin content in the ripe cheese mcg per 100 g
b – Difference in the vitamin content of unripened and ripe cheese mcg per 100 g
c – $\frac{\Delta \text{Vit.}}{\Delta \text{Vit. min}}$

TABLE 21

CHEMICAL PROPERTIES OF THE CHEESE MASS RIPENING IN EFFECT OF VARIOUS PENICILLIUM ROQUEFORTI STRAINS ACTION

Penic. roquef Symbol	pH of cheese	Total protein %	Soluble protein %	Soluble protein in % proport. to tot. prot.	Water cont. %	Fat acidity	Peroxide number	TBA index %
2	7.5	25.19	11.86	47.1	47.34	42.1	1.5	94
2/1	7.1	21.34	11.63	54.5	51.56	145.5	27.3	25
2/2	6.85	21.87	14.75	67.4	50.41	248.6	18.0	70
2/3	7.35	23.76	12.64	53.2	49.09	155.4	15.3	43
2/4	6.9	22.99	13.55	58.1	51.1	76.8	6.6	85
2/5	7.35	33.88	13.57	40.1	49.5	146.0	22.4	30
7	7.25	20.8	12.77	61.2	49.53	28.8	0.41	91
7/1	7.15	21.75	13.41	61.7	50.48	106.8	8.8	85
7/2	6.7	15.7	12.76	81.3	48.37	136.6	6.1	80
7/3	7.2	20.62	12.29	59.6	49.12	68.6	18.1	62
7/4	7.35	21.3	13.05	61.3	48.00	170.0	24.0	52
7/5	7.05	16.87	12.76	75.6	51.14	163.8	13.5	50

stronger lipolytic properties which, among others, are visible in its ability for fat hydrolisis and its oxidative abilities as well.

The mutant "2/2" is able to hydrolize the fat at a very great velocity, much greater than the mutant "7/4", but its oxidative abilities are apparently lower.

It may be generally stated that in effect of mutagenic factors action the lipolytic properties can be induced to a greater extent, whereas the proteolytic abilities of strains remain rather unchanged.

The mean values from determinations of the B-group vitamin content in the ripened cheese mass are presented in Table 21.

It should be emphasized that the mutant "2/2" distinguishes itself from among the others by its ability to biosynthesize some vitamins of the B-group. This may be related especially to thiamine, riboflavin and pantothenic acid. The mutant is also securing a greater content of niacin if compared with the parent strain.

As the second may be listed the *Penicillium candidum* mutant "2/5" able to produce considerable amounts of niacin and biotin.

The above two mutants can be recommended for application in manufacture of the Roquefort-type cheese. It also deserves to be stressed that in the course of further investigations these two mutants have shown an apparent durability of their biochemical properties.

IV. DISCUSSION

The investigations performed have shown that there exist essential differences between the separate strains of *Penicillium candidum* and *Penicillium roqueforti* as to their abilities for biosynthesis of thiamine, riboflavin, niacin, pantothenic acid, biotin and cobalamin.

On the ground of evaluation of their technological utility (e.g. by means of experimental cheese manufactures with the use of different strains and also of their organoleptic testing), biochemical properties (mainly those proteolytic and lipolytic) and of their abilities to produce vitamins the two most appropriate strains of *Penicillium candidum* and *Penicillium roqueforti* have been respectively selected. These were the following: *Penicillium candidum Nos 2* and *10*, and *Penicillium roqueforti Nos 2* and *7*. Their usefulness for practical applications has been confirmed in the series of experimental cheese manufactures.

The attempts to evaluate the effect of some factors on the ability of *Penicillium candidum* to biosynthesize vitamins have pointed to

(a) the essentiality of the culture's age for its vitaminogenic ability (the shapes of curves illustrating this phenomenon have been found specifically characteristic for each one of vitamins);

(b) the composition of medium as an important differentiating factor, what may be clearly seen from a comparison of curves depicting the changes in the Capek-Dox medium and in hydrolized milk (cf. Figure 4, Diagrams B and D);

(c) an important role of pH of medium, especially in biosynthesis of niacin (it has been found that the optimum pH value is a little above 6.0);

The above-mentioned factors and the relatively great differences in vitamin contents observed in the course of successive experimental cheese manufactures by the use of the same strain of *Penicillium candidum* point to an important role played by the manufacturing conditions with regard to the final content of vitamins. In this connection it becomes apparent that it would be a most purposeful task to undertake a special research with the aim to establish the optimum manufacturing conditions securing a high biological quality of the mold-ripened cheese. The need to undertake this research results from an ascertainment that the vitamin content in cheese is equally depending

TABLE 22

ABILITY TO PRODUCE VITAMINS IN MUTANTS OF PENICILLIUM ROQUEFORTI IN THE CHEESE MASS

Vitamin	Strain /mutant/ of Penicillium roqueforti No											
	2	2/2	2/3	2/3	2/4	2/5	7	7/1	7/2	7/3	7/4	7/5
B_1 mcg per 100 g	132.5	120.5	320.6	131.3	126.8	147.5	115.0	115.5	98.3	137.5	110.0	100.5
B_2 mcg per 100 g	728	947	1210	1025	931	921	948	1146	1055	1038	858	800
PP mcg per 100 g	2237	2299	2451	2208	1972	3077	2429	2197	2845	2876	2824	2875
Pant. acid mcg per 100 g	893	880	1283	885	800	1053	1050	1008	992	957	850	790
Biotin mcg per 100 g	12.1	11.5	11.4	10.5	8.6	12.9	9.8	6.2	9.1	11.1	8.7	12.2
B_{12} mcg per 100 g	1.32	1.15	1.21	0.97	1.05	1.30	1.17	1.06	1.09	1.06	0.93	1.09

upon the biological and technological factors. Attempts to obtain mutants under the action of U.V.-, X-, and gamma-rays have led to the following observations:

(a) that the above-mentioned mutagenic agents in most cases are causing a decrease in the ability of a mutant obtained to biosynthesize vitamins (only a very small part from among the irradiated spores distinguished itself by an increased vitality if compared with the parent strain);

(b) that it has proved possible to obtain biochemical mutants differing from their parent strains mainly by their lipolytic properties (it is possible relatively seldom to obtain mutants with their proteolytic properties changed);

(c) that the lethal effect of U.V.-, X-, and gamma-rays on the *Penicillium candidum* spores is smaller than that on the *Penicillium roqueforti* spores;

(d) that the obtained mutants of *Penicillium candidum* show a certain variability of their vitamino-genic abilities in the course of successive inoculations (this becomes apparent by a decrease of vitamin content in cheese manufactured by the use of mutants after about 1-year storage);

(e) that the chosen mutants of *Penicillium roqueforti* are showing greater durability than that of chosen mutants of *Penicillium candidum.*

As a practical result of investigations perfomed it may be recommended to apply in manufacture of Camembert cheese the chosen strains of *Penicillium candidum Nos 2* and *10.* The mutants obtained from these strains cannot, however, be recommended for this purpose in view of the variability of their properties.

For production of Blue cheese from among the strains of *Penicillium roqueforti* may be recommended an application of a mixture composed of the strain No 7 and the mutants Nos "2/2" and "2/5".

LITERATURE

(1) Adrian J. Les dosage microbiologique des vitamins du groupe B; Les cahiers Techniques du CNRS, 1959.
(2) Antoniani G. Mondo del Latte 1951, 334.
(3) Brochu E., Riel R., Vezina C., Les bacteries lactiques et les lait fermentés (monographie), Ministry of Agriculture Québeck 1959.
(4) Burkholder P.R., Collieré J., Moyer D.: Food Res. 1943, 8, 314.
(5) Cailleau R., Adrian J.M., Levy J.: Ann. Agr. 1949, 3, 443.
(6) Callieri D.A.: Acta chimica Scand. 1959, 13, 737.
(7) Claveau J.: Ind. Agricol. Alim. 1956, 73, 3.
(8) Costavassilis C.: Rev. Pathol. Gen. et. Physiol. Clin. 1958, 58, 297.
(9) Damil L., Norris L.C.: Food Res. 1944, 9, 312.
(10) Davidov R.B., Gulko L.E.: Mol. Prom. 1951, 12, 28.
(11) Dłużewski M., Pijanowski E., Zmarlicki S.: Roczniki Technologii i Chemii Żywności 1961, VII, 127.
(12) Federico L., Valle T.: Ann. Speriment. Agri. Ital. 1954, 8, 1489.
(13) Haudiniére A.: Le Lait, 1950, 30, 37.
(14) Irvine O.R., Bryant L.R., Sproule W.H., Jackson H., Crook A., Johnstone W.M.: Sci. Agr. 1945, 25, 845.
(15) Karlin R.: Ann. Nutr. et. Alim. 1957, 11, 91.
(16) Karlin R.,: Int. Z. Vitaminoforsch., 1961, 31, 2, 176.
(17) Karlin R.,: Le Lait, 1962, 42, 241 and 415.
(18) Lea: Ztsch. Leb. Unt.: 1937, 73, 278.
(19) Leuthardt F.: Lehrbuch der Physiologischen Chemie. W. de Gruytar a. Co/Berlin 1959, 783.
(20) Lie and Lunde: Nord. Med. 1940, 8, 2250./quoted after J.D.Res. 1949, 16, 91/
(21) Mašek J., Maxa V., Teplý M.: Kontrola jakosti mlékařských kultur a zakysů 1960, 154.
(22) Monzini A., Artrom A.: Il Latte 1954, 28, 77.
(23) Patton a. Kurtz: J. Dairy Sci: 1955, 38, 401.
(24) Randoin L.: Bull. Soc. Chim. Biol. 1941, 23, 90.
(25) Randoin L., Causeret J.: Le Lait, 1958, 38, 43.
(26) Ritter W.: Schweiz. Milchztg. 1944, 16/17, 1.
(27) Ritter W.: Schweiz. Milchztg. 1944, 70, 71.
(28) Ritter W.: Rev. Suisse Path. Backer. 1950, 13, 547.
(29) Roerlich M.: Z. Lebens, Unt. u. Forsch. 1954, 99, 109.
(30) Sabry Z.I., Gurerant N.B.: J. Dairy Sci. 1958, 41, 925.
(31) Shahani K.M., Hathaway I.L. a. Kelly P.L. — J. Dairi Sci; 1962, 45, 7, 833.
(32) Snell E.E., Strong F.M.: Ind. Eng. Chem. Anal. Ed. 1939, 11, 346
(33) Sullivan R., Bloow E., Jarmol J.: J. Nutrition 1943, 25, 463.
(34) Zook E.S., Mc. Arthur., Toepper E.W.: U.S. Sept. Agric. Handbook 1956, 97, 1.
(35) Techniques de Laboratoire 1.II.1954. p. 44. Masson, Paris.